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PATENT & TRADEMARK OFFICE

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TRANSMITTAL OF APPEAL BRIEF	Docket No. AH-CLFR:181USD5

In re Application of: David H. Walker et al.

Application No.	Filing Date	Examiner	Group Art Unit
10/731,554-Conf. #6350	December 9, 2003	P. Baskar	1645

Invention: HOMOLOGOUS 28-KILODALTON IMMUNODOMINANT PROTEIN GENES OF EHRLICHIA CANIS AND USES THEREOF

TO THE COMMISSIONER OF PATENTS:

Transmitted herewith is the Appeal Brief in this application, with respect to the Notice of Appeal filed: October 2, 2006 .

The fee for filing this Appeal Brief is $ 250.00 .

- [ ] Large Entity
- [x] Small Entity

- [ ] A petition for extension of time is also enclosed.

The fee for the extension of time is ________ .

- [ ] A check in the amount of $ 250.00 is enclosed.
- [x] Charge the amount of the fee to Deposit Account No. 06-2375 . This sheet is submitted in duplicate.
- [ ] Payment by credit card. Form PTO-2038 is attached.
- [x] The Director is hereby authorized to charge any additional fees that may be required or credit any overpayment to Deposit Account No. 06-2375 . This sheet is submitted in duplicate.

Dated: December 1, 2006

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Appeal Brief Transmittal

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV 678186560, on the date shown below in an envelope addressed to: MS Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: December 1, 2006 Signature: ________ (Monica T. Owens)

PTO/SB/17 (07-06)
Approved for use through 01/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2006

[x] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 250.00

Complete if Known	
Application Number	10/731,554-Conf. #6350
Filing Date	December 9, 2003
First Named Inventor	David H. Walker
Examiner Name	P. Baskar
Art Unit	1645
Attorney Docket No.	AH-CLFR:181USD5

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): ______

[x] Deposit Account Deposit Account Number: 06-2375 Deposit Account Name: Fulbright & Jaworski L.L.P.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[ ] Charge fee(s) indicated below
[•] Charge fee(s) indicated below, **except for the filing fee**
[x] Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17
[x] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	Fee ($)	Small Entity Fee ($)	Fee ($)	Small Entity Fee ($)	Fee ($)	Small Entity Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims ______ - 20 = Extra Claims ______ x Fee ($) ______ = Fee Paid ($) ______
HP = highest number of total claims paid for, if greater than 20.

Indep. Claims ______ - 3 = Extra Claims ______ x Fee ($) ______ = Fee Paid ($) ______
HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims
Fee ($) ______ Fee Paid ($) ______

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets ______ - 100 = Extra Sheets ______ /50 Number of each additional 50 or fraction thereof ______ (round up to a whole number) x Fee ($) ______ = Fee Paid ($) ______

4. OTHER FEE(S) Fees Paid ($)

Non-English Specification, $130 fee (no small entity discount)

Other (e.g., late filing surcharge): 2402 Filing a brief in support of an appeal 250.00

SUBMITTED BY

Signature	[signature]	Registration No. (Attorney/Agent)	45,579	Telephone	(713) 651-3735
Name (Print/Type)	Melissa L. Sistrunk			Date	December 1, 2006

Fee Transmittal

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV 678186560US, on the date shown below in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: December 1, 2006 Signature: Monica Owens (Monica T. Owens)

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PATENT & TRADEMARK OFFICE

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Dated: December 1, 2006 Signature: (Monica T. Owens)

Docket No.: AH-CLFR:181USD5
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
David H. Walker et al.

Application No.: 10/731,554

Filed: December 9, 2003 — Art Unit: 1645

For: HOMOLOGOUS 28-KILODALTON IMMUNODOMINANT PROTEIN GENES OF EHRLICHIA CANIS AND USES THEREOF — Examiner: Baskar, P.

APPEAL BRIEF

Commissioner for Patents
Washington, D.C. 20231

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
David H. Walker et al.

Application No.: 10/731,554

Filed: December 9, 2003

Art Unit: 1645

For: HOMOLOGOUS 28-KILODALTON IMMUNODOMINANT PROTEIN GENES OF EHRLICHIA CANIS AND USES THEREOF

Examiner: Baskar, P.

APPEAL BRIEF

MS Appeal Brief
Commissioner of Patents
Washington, D.C. 20231

Sir:

Appellants hereby submit an Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated June 30, 2006 (the "Action"). The Notice of Appeal was filed on October 2, 2006.

The fee for filing this Appeal Brief is $250.00. Appellants assert that an additional fee is not required, but if this is in error, please charge the Deposit Account 06-2375 under the reference number AH-CLFR:181USD5, from which the undersigned is allowed to withdraw.

Please date stamp and return the attached postcard as evidence of receipt.

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12/05/2006 DEMMANU1 00000028 062375 10731554
01 FC:2402 250.00 DA

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Research Development Foundation.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-20 are canceled. Claims 21-23 are under examination and are the subject of appeal.

IV. STATUS OF AMENDMENTS

There are no amendments.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention generally concerns methods of inhibiting or preventing *Ehrlichia canis* infection in a subject by administering a polypeptide of SEQ ID NO:46 to the subject prior to exposure or to a subject suspected of being exposed to or suspected of being infected by *E. canis*, as represented by claim 21 and that finds support in the specification at least in the original claims, the sequence listing, and at paragraphs **[0016]** and **[0053]**, for example. In particular embodiments, SEQ ID NO:46 is encoded by a polynucleotide of SEQ ID NO:45, as represented in claim 22 and that finds support in the specification at least in the original claims, the sequence listing, and at paragraphs **[0016]** and **[0046]**, for example. In further embodiments, SEQ ID NO:46 is dispersed in a pharmaceutically acceptable carrier, as represented in claim 23 and that finds support in the specification at least in the original claims at paragraphs **[0016]** and **[0062]**, for example.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 21-23 are rejected under 35 U.S.C. §112, second paragraph, for being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter of the invention.

Claims 21-23 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Ohashi *et al.*, 1998 (Infec. Immun. 66; 132-139) in view of Ohashi *et al.*, 1998 (J. Clin. Microbiol, 2671-2680).

Claims 21-23 were rejected under 35 U.S.C. §112, first paragraph, for allegedly failing to comply with the written description requirement.

VII. ARGUMENT

A. Substantial Evidence Required to Uphold the Examiner's Position

As an initial matter, Appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Issues under 35 U.S.C. §112, second paragraph

Claims 21-23 were rejected under 35 U.S.C. §112, second paragraph for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. Appellants respectfully disagree.

In particular, the Examiner states that the limitation "preventing" has no support in the specification, but as discussed in Section **VII. D.** below, prevention is referred to in two separate texts in the application.

Further, the Examiner still appears to be confused by the terminology of the claim regarding identifying a subject prior to exposure or suspected of being exposed to or suspected of being infected with *Ehrlichia canis*. Specifically, the Examiner stated in the Office Action dated January 11, 2006, and referred to again in the final Action, that the claim was vague and the Examiner found it unclear how to inhibit *E. canis* infection in a subject prior to exposure or suspected of being exposed to an infection when there is no infection to begin with.

Claim 21 encompasses a method for inhibiting or preventing infection by identifying a subject prior to exposure with *E. canis* <u>or</u> a subject suspected of being exposed to *E. canis* <u>or</u> a subject suspected of being infected with *E. canis*, and then administering the protein. The Examiner considers it unclear how to inhibit *E. canis* infection in a subject prior to exposure or suspected of being exposed to an infection because there is no infection to begin with. Appellants reiterate that an infection can be inhibited by inhibiting its onset, and therefore the claim is not indefinite.

Appellants respectfully request reversal of the rejection.

C. Issues under 35 U.S.C. §103(a)

Claims 21-23 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Ohashi *et al.*, 1998 (Infec. Immun. 66; 132-139) ("Ohashi C24," previously referred to

by Appellants as "Ohashi A") in view of Ohashi *et al.*, 1998 (J. Clin. Microbiol., 2671-2680) ("Ohashi C23," previously referred to by Appellants as "Ohashi B"). Appellants respectfully disagree.

The Examiner has failed to make a *prima facie* case of obviousness, because all elements of the claims are not taught or suggested in the combination of Ohashi C24 and Ohashi C23. The claims generally concern methods of inhibiting or preventing *E. canis* infection in an individual by administering a composition comprising a polypeptide of SEQ ID NO:46 in an amount effective to inhibit *Ehrlichia canis* infection. Ohashi C23 concerns ***serodiagnosis*** of *E. canis* by assaying for one of three p30 kDa outer membrane proteins, none of which are SEQ ID NO:46. Ohashi C24 concerns identification and characterization of p28 kDa proteins in *E. chaffeensis* and includes protection against *E. chaffeensis* challenge in rP28-immunized mice. It is not obvious to employ a p30 *E. canis* protein for inhibiting infection when the proteins are described as being serodiagnostic, nor is it obvious to use an *E. chaffeensis* p28 protein to inhibit an *E. canis* infection. The person of ordinary skill in the art is an objective legal construct presumed to think along conventional lines without undertaking to innovate, whether by systematic research or by extraordinary insights, (*Life Technologies, Inc. v. Clontech Laboratories, Inc.*, 224 F.3d 1320, 56 U.S.P.Q.2d 1186 (Fed. Cir. 2000), citing *The Standard Oil Co. v. American Cyanamid Company,* 774 F.2d 448, 227 U.S.P.Q. 293 (Fed. Cir. 1985)), so there would be no suggestion or motivation to employ the p30 proteins of *E. canis* in Ohashi C23 for any purpose other than serodiagnosis in that very same organism. Therefore, there is no suggestion or motivation to utilize the p30 *E. canis* proteins for immunoprotection against *E. canis*.

The Ohashi C23 reference solely concerns serodiagnosis of *E. canis* and not immunoprotection from *E. canis*. For example, in Ohashi C23 at pp. 2673-2674, the authors describe identification of three p30 kDa proteins: P30, P30-1, and P30a. Ohashi then

describes optimum dilutions for the antiserum for serodiagnosis (pp. 2676-2677) and use of rP30 antigens for examination of dog plasma. Nowhere in Ohashi C23 is there teaching or suggestion to use P30, P30-1, or P30a for inhibiting *E. canis* infection.

In addition, Ohashi C23 does not teach or suggest SEQ ID NO:46 itself. Although the text of Ohashi C23 refers to the particular sequences of P30, P30-1, and P30a in Figure 2, none of these sequences teach or suggest SEQ ID NO:46. On p. 2673, Ohashi C23 also refers to the sequences in GenBank® accession numbers AF078553, AF078554, and AF078555 (right column). However, at the time of filing none of these GenBank® sequences described or suggested SEQ ID NO:46 (see Exhibits 3, 4, and 5). If anything, Ohashi C23 teaches away from employing SEQ ID NO:46 because it concerns p30 proteins that are dissimilar with SEQ ID NO:46. While an updated version of AF078553 (Exhibit 3) appears to have a sequence that is similar to SEQ ID NO:46, this sequence was not disclosed until after Appellants' filing date (see the date of April 2, 2001 on AF078553 in Exhibit 3). Therefore, SEQ ID NO:46 was not known at the time of filing of the application.

Turning now to Ohashi C24, the reference teaches immunoprotection for *E. chaffeensis* with *E. chaffeensis* p28 proteins, and this reference does not teach, suggest, or provide motivation for use of SEQ ID NO:46 for immunoprotection for *E. canis*. It is noted that *E. chaffeensis* and *E. canis* are different organisms. The reference discloses identification of multiple major outer membrane proteins of *E. chaffeensis* (p. 133-134) and characterizes the proteins (p. 134-137), including demonstration of protection against *E. chaffeensis* challenge in rP28-immunized mice. In the background of Ohashi C24, the authors refer to other articles that showed cross-reactivity between *E. chaffeensis* and *E. canis* 28-30 kDa proteins. However, nowhere in Ohashi C24 does the reference teach or suggest use of any *E. canis* protein to inhibit *E. canis* infection, and it certainly does not teach or suggest use of Appellants' particular SEQ ID NO:46 to inhibit *E. canis* infection.

The Examiner contends that Appellants' claimed invention is made obvious over the combination of Ohashi C23 and Ohashi C24, because Ohashi C24 refers to cross-reactivity between p28/p30 proteins of *E. canis* and *E. chaffeensis*, but Appellants assert that there is no suggestion or motivation to make the combination. Even if Ohashi C24 did suggest that one could employ p30 proteins of *E. canis* for immunoprotection, there is no teaching or suggestion that any *E. chaffeensis* antibodies cross-react with *E. canis* SEQ ID NO:46. Moreover, one of skill in the art is taught by Ohashi C23 that the *E. canis* p30 proteins are useful for serodiagnosis, so one of skill in the art would be led away from Ohashi C23 for use of the proteins for immunoprotection against *E. canis*. Furthermore, even if Ohashi C24 suggested utilizing one of the p30 proteins of *E. canis* in Ohashi C23 for immunoprotection, this would teach or suggest to one of skill in the art to employ one of the p30 proteins described in Ohashi C23 that were known at the time and not the unknown SEQ ID NO:46. This also would lead away from Appellants' claimed invention. That is, even if it is obvious from Ohashi C24 to try some *E. canis* protein for inhibiting infection, it is not obvious to utilize Appellants' specific SEQ ID NO:46, particularly when a variety of non-identical sequences to SEQ ID NO:46 were referred to in Ohashi C23 and SEQ ID NO:46 was unknown at the time of filing. Therefore, methods to inhibit infection with any *E. canis* p30 protein, and in particular SEQ ID NO:46, were not taught or suggested in the combination of Ohashi C23 and Ohashi C24, and there is no *prima facie* case of obviousness.

Appellants respectfully request reversal of the rejection.

D. Issues under 35 U.S.C. §112, first paragraph

Claims 21-23 were rejected under 35 U.S.C. §112, first paragraph, for allegedly failing to comply with the written description requirement.

The Examiner sets forth a written description rejection for subject matter that is considered new matter. In particular, the Examiner states that there is new matter because the

limitation in claim 21 of "preventing" is not supported in the specification. Appellants assert that there is no new matter, because in two different texts of the specification Appellants address prevention. In particular, the Abstract states that the proteins are, "...useful in the development of vaccines and serodiagnostics that are particularly effective for disease ***prevention*** and serodiagnosis" (emphasis added). Furthermore, in paragraph **[0124]**, it states the following: "The conservation of p28 genes in *E. canis* isolates may provide an opportunity to develop vaccine and serodiagnostic antigens that are particularly effective for disease ***prevention*** and serodiagnosis" (emphasis added).

Therefore, the term "preventing" in claim 21 does not introduce new matter, and Appellants respectfully request reversal of the rejection.

VIII. CONCLUSION

Appellants have provided arguments that overcome the pending rejection. Appellants respectfully submit that the Office Action's conclusions that the claims should be rejected are unwarranted. It is therefore requested that the Board overturn the Action's rejections.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Dated: Dec. 1, 2006

Respectfully submitted,

By [signature]

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APPENDIX 1

CLAIMS ON APPEAL

21. A method of inhibiting or preventing *Ehrlichia canis* infection in a subject comprising the steps of:

identifying a subject prior to exposure or suspected of being exposed to or infected with *Ehrlichia canis*; and

administering a composition comprising a polypeptide of SEQ ID NO:46 in an amount effective to inhibit *Ehrlichia canis* infection.

22. The method of claim 21, wherein said SEQ ID NO:46 is encoded by a polynucleotide of SEQ ID NO:45.

23. The method of claim 21, wherein said SEQ ID NO:46 is dispersed in a pharmaceutically acceptable carrier.

APPENDIX 2

EVIDENCE APPENDIX

Exhibit 1. Ohashi *et al.* (Infec. Immun., 66:132-139, 1998) made of record in the Office Action mailed January 11, 2006

Exhibit 2. Ohashi et al. (J. Clin. Microbiol., 2671-2680, 1998) made of record in the Office Action mailed January 11, 2006

Exhibit 3. National Center for Biotechnology Information, GenBank Accession No. AF078553, GenBank database; April 2, 2001, made of record in the Response and Supplemental IDS filed March 29, 2006

Exhibit 4. National Center for Biotechnology Information, GenBank Accession No. AF078554, GenBank database; October 26, 1998, made of record in the Response and Supplemental IDS filed March 29, 2006

Exhibit 5. National Center for Biotechnology Information, GenBank Accession No. AF078555, GenBank database; October 26, 1998, made of record in the Response and Supplemental IDS filed March 29, 2006

APPENDIX 3

RELATED PROCEEDINGS APPENDIX

NONE

C24

INFECTION AND IMMUNITY, Jan. 1998, p. 132–139
0019-9567/98/$04.00+0

Vol. 66, No. 1

Immunodominant Major Outer Membrane Proteins of *Ehrlichia chaffeensis* Are Encoded by a Polymorphic Multigene Family

NORIO OHASHI, NING ZHI, YILAN ZHANG, AND YASUKO RIKIHISA*

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Received 21 January 1997/Returned for modification 24 March 1997/Accepted 8 October 1997

Several immunodominant major proteins ranging from 23 to 30 kDa were identified in the outer membrane fractions of *Ehrlichia chaffeensis* and *Ehrlichia canis*. The N-terminal amino acid sequence of a 28-kDa protein of *E. chaffeensis* (one of the major proteins) was determined. The gene (*p28*), almost full length, encoding the 28-kDa protein was cloned by PCR with primers designed based on the N-terminal sequence of the *E. chaffeensis* 28-kDa protein and the consensus sequence between the C termini of the *Cowdria ruminantium* MAP-1 and *Anaplasma marginale* MSP-4 proteins. The *p28* gene was overexpressed, and antibody to the recombinant protein was raised in a rabbit. The antibody and serum from a patient infected with *E. chaffeensis* reacted with the recombinant protein, three proteins (29, 28, and 25 kDa) of *E. chaffeensis*, and a 30-kDa protein of *E. canis*. Immunoelectron microscopy with the rabbit antibody revealed that the antigenic epitope of the 28-kDa protein was exposed on the surface of *E. chaffeensis*. Southern blot analysis with a ^{32}P-labeled *p28* gene probe revealed multiple copies of genes homologous to *p28* in the *E. chaffeensis* genome. Six copies of the *p28* gene were cloned and sequenced from the genomic DNA by using the same probe. The open reading frames of these gene copies were tandemly arranged with intergenic spaces. They were nonidentical genes and contained a semivariable region and three hypervariable regions in the predicted protein molecules. One of the gene copies encoded a protein with an internal amino acid sequence identical to the chemically determined N-terminal amino acid sequence of a 23-kDa protein of *E. chaffeensis*. Immunization with the recombinant P28 protein protected mice from infection with *E. chaffeensis*. These findings suggest that the 30-kDa-range proteins of *E. chaffeensis* represent a family of antigenically related homologous proteins encoded by a single gene family.

Ehrlichia chaffeensis, which causes human monocytic ehrlichiosis, is an obligate intracellular bacterium of monocytes and macrophages and belongs to the family *Rickettsiaceae*. Human ehrlichiosis is a tick-borne illness and was first reported in 1987 in the United States (21). Most patients have fever, chills, headache, arthralgia, myalgia, and hematologic abnormalities, including thrombocytopenia and leukopenia. Elevation of liver enzymes occurs in most patients. Since 1987, over 400 cases of human ehrlichiosis, detected primarily by serological means, have been reported in 30 states (3, 14, 16).

Recently, several protein antigens of *E. chaffeensis* were identified by Western blot analysis with naturally infected human sera, experimentally inoculated dog sera, or monoclonal antibodies (7–10, 13, 30, 35, 40–42). Two of these antigens, namely, a heat shock protein (HSP) 60 homolog (35) and a 120-kDa protein (41, 42), have been cloned, sequenced, and expressed. Two *E. chaffeensis* proteins ranging from 28 to 30 kDa were shown to be dominant antigens and were cross-reactive between two *Ehrlichia* spp.: *E. chaffeensis* and *E. canis* (7, 30). Studies with monoclonal antibodies (MAbs) against *E. chaffeensis* showed that two or three proteins of from 22 to 30 kDa react with three MAbs by Western blotting and that these antigens are exposed on the surface of the organism as determined by immunogold labeling of negatively staining ehrlichiae (8–10, 40). However, why multiple proteins of different molecular sizes react with the MAbs has not been answered. These *E. chaffeensis* antigens in the 30-kDa range have not been examined at the molecular level.

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In this study, we demonstrated that a potentially immunoprotective 28-kDa protein (designated P28) located on the *E. chaffeensis* surface and antigenically cross-reactive proteins in the 30-kDa range are encoded by a multigene family.

MATERIALS AND METHODS

Organisms and purification. The *E. chaffeensis* Arkansas strain and *E. canis* Oklahoma strain were cultivated in the DH82 dog macrophage cell line (30) and purified by Percoll density gradient centrifugation as described elsewhere (32, 38).

Preparation of the ehrlichial outer membrane fraction. The procedure for *Orientia tsutsugamushi* was followed, with modifications (25). Briefly, purified ehrlichiae (100 μg) were suspended with 10 mM sodium phosphate buffer (pH 7.4) containing 0.1% sodium *N*-lauroyl sarcosine (Sarkosyl) (Sigma, St. Louis, Mo.), 50 μg (each) of DNase I (Sigma) and RNase A (Sigma) per ml, and 2.5 mM $MgCl_2$. After incubation at 37°C for 30 min, the sample was separated by centrifugation at 10,000 × *g* for 1 h into the soluble supernatant and the insoluble precipitate. The insoluble pellet was resuspended two or three times with 0.1% Sarkosyl and centrifuged. The final pellet was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described elsewhere (31) and by electron microscopy. The pellet was used as the ehrlichial outer membrane fraction. To investigate contamination by the ehrlichial inner membrane, succinic dehydrogenase activity was examined as described elsewhere (11).

Analysis of the N-terminal amino acid sequences of outer membrane proteins in the 30-kDa range. Proteins in the Sarkosyl-insoluble pellet prepared from 400 μg of purified *E. chaffeensis* were separated by reversed discontinuous SDS-PAGE (RdSDS-PAGE) (a 2.5-cm-long 17% gel on top of an 11-cm-long 12% gel) and electrophoretically transferred to a ProBlot membrane (Applied Biosystems, Foster City, Calif.) as described elsewhere (44). The portion of the membrane containing bound proteins was excised and analyzed with an Applied Biosystems protein sequencer (model 470).

Primer design for amplification of a gene (*p28*) encoding a 28-kDa major protein (P28) of *E. chaffeensis*. The N-terminal amino acid sequence of P28 (one of the major proteins separated by RdSDS-PAGE as described above) was determined as DPAGSGINGNFYSGKYMP. We designed a forward primer, FECH1, based on amino acids 6 to 12 of this sequence: 5'-CGGGATCCGAATTCGG(A/T/G/C)AT(A/T/C)AA(T/C)GG(A/T/G/C)AA(T/C)TT(T/C)TA-3'. Amino acids at positions 1 to 5 of the N terminus of P28 were not included in this primer design to

increase annealing efficiency, since Ser with six codons was present at position 5. For insertion into an expression vector, a 14-bp sequence (underlined) was added at the 5′ end of the primer to create an *Eco*RI site and a *Bam*HI site.

A reverse primer was designed from two proteins which we found to be related to P28 based on N-terminal amino acid sequence comparison. One of the proteins was *Cowdria ruminantium* major antigen protein 1 (MAP-1). The C-terminal sequence of MAP-1 is as follows: (N terminus) . . . GGRFVF* (C terminus) (* is the termination codon) (36). The other protein was the *Anaplasma marginale* major surface protein 4 (MSP-4) (23), the entire amino acid sequence of which is homologous to that of *C. ruminantium* MAP-1 (36). The C-terminal sequence of MSP-4 is as follows: (N terminus) . . . GARFLFS* (C terminus). An oligonucleotide primer, RECH2, complementary to a DNA sequence corresponding to the amino acid sequence conserved between the C termini of MAP-1 and MSP-4, (N terminus) G(G/A)RF(V/L)F* (C terminus), was prepared, with the addition of a 9-bp sequence (underlined) including a *Not*I site at the 5′ end for ligation into an expression vector: 5′-AGCGGCCGCTTA(A/G)AA(T/C)A(C/G)(A/G)AA(C/T)CTT(C/G)CTCC-3′.

Cloning, sequencing, and expression of the *p28* gene. Genomic DNA of *E. chaffeensis* was isolated from purified organisms as described elsewhere (24). PCR amplification with FECH1 and RECH2 primers was performed with a Perkin-Elmer Cetus DNA Thermal Cycler (model 480). A 0.8-kb amplified product was cloned in the pCRII vector of a TA cloning kit, as described by the manufacturer (Invitrogen Co., San Diego, Calif.). The clone obtained was designated pCRII*p28*. Both strands of the inserted DNA were sequenced by a dideoxy termination method with an Applied Biosystems 373A DNA sequencer.

The 0.8-kb *p28* gene was excised from the clone pCRII*p28* by *Eco*RI-*Not*I double digestion, ligated into *Eco*RI-*Not*I sites of a pET 29a expression vector, and amplified in *Escherichia coli* BL21(DE3)pLysS (Novagen, Inc., Madison, Wis.). The clone (designated pET29*p28*) produced a fusion protein with a 35-amino-acid sequence carried from the vector at the N terminus.

Antisera and Western blot analysis. Convalescent-phase serum from a patient with clinical signs of human ehrlichiosis was used as described previously (30). For preparation of the rabbit anti-recombinant P28 (anti-rP28) antibody, the gel band corresponding to rP28 in SDS-PAGE was excised without staining, minced in phosphate-buffered saline (PBS) (pH 7.4), and mixed with an equal volume of Freund's incomplete adjuvant (Sigma). The mixture (1 mg of protein each time) was subcutaneously injected into a rabbit every 2 weeks for four times. Antibody titers of the patient serum and the rabbit anti-rP28 antibody against *E. chaffeensis* antigen were determined to be 1:2,560 and 1:1,280, respectively, by indirect immunofluorescence assay as described elsewhere (29).

Western blot analyses were performed with 1:1,000 dilutions of these sera by a procedure described elsewhere (31). The rabbit anti-rP28 antibody was preabsorbed twice with pET29a-transformed *E. coli* at 37°C for 1 h each at a 1:300 dilution prior to use. Alkaline phosphatase-conjugated affinity-purified anti-human or anti-rabbit immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used at a 1:1,000 or 1:2,000 dilution as a secondary antibody.

Immunoelectron microscopy. *E. chaffeensis*-infected DH82 cells were sonicated and centrifuged at 400 × *g* for 10 min. The supernatant was then centrifuged at 10,000 × *g* for 10 min to obtain an ehrlichia-enriched pellet. The pellet was resuspended and incubated with rabbit anti-rP28 antibody or normal rabbit serum (1:100 dilution) at 37°C for 1 h in PBS containing 1% bovine serum albumin. After being washed, the ehrlichiae were incubated with gold-conjugated protein G (20 nm; Sigma) at a 1:30 dilution for 1 h at room temperature in PBS containing 1% bovine serum albumin. After being washed again, the specimen was fixed with 1.25% formaldehyde, 2.5% glutaraldehyde, and 0.03% trinitrophenol in 0.1 M cacodylate buffer (pH 7.4) for 24 h and postfixed in 1% osmium–1.5% potassium ferricyanide for 1 h (34). The section was then embedded in PolyBed 812 (Polysciences, Warrington, Pa.). The specimen was ultrathin sectioned at 60 nm, stained with uranyl acetate and lead citrate, and observed with a Philips 300 transmission electron microscope at 60 kV.

Southern blot analysis. Genomic DNA extracted from the purified *E. chaffeensis* (200 ng) was digested with restriction endonucleases, electrophoresed, and transferred to a Hybond-N$^+$ nylon membrane (Amersham, Arlington Heights, Ill.) by a standard method (33). The 0.8-kb *p28* gene fragment from the clone pCRII*p28* was labeled with [α-^{32}P]dATP by the random primer method by using a kit (Boehringer Mannheim, Indianapolis, Ind.), and the labeled fragment was used as a DNA probe. Hybridization was performed at 60°C in rapid-hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in 0.1× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) with 1% SDS at 55°C, and the hybridized probes were exposed to Hyperfilm (Amersham) at −80°C.

Cloning and sequencing of genomic copies of the *E. chaffeensis p28* gene. The *Eco*RI and *Pst*I fragments of DNA, detected by genomic Southern blot analysis as described above, were inserted into pBluescript II KS(+) vectors, and the recombinant plasmids were introduced into *E. coli* DH5α. By using the colony hybridization method (33) with the ^{32}P-labeled *p28* gene probe, four positive clones were isolated from the transformant. The positive clones were designated pEC2.6, pEC3.6, pPS2.6, and pPS3.6. These contained the ehrlichial DNA fragments of 2.6 (*Eco*RI), 3.6 (*Eco*RI), 2.6 (*Pst*I), and 3.6 (*Pst*I) kb, respectively. The inserts of the clones pEC3.6 and pPS2.6 overlapped as shown in Fig. 6. The overlapping area was further confirmed by PCR of *E. chaffeensis* genomic DNA with two pairs of primer sets interposing the junctions of the four clones (see Fig. 6). The 1.1- to 1.6-kb *Hin*dIII-*Hin*dIII, *Hin*dIII-*Eco*RI, or *Xho*I-*Eco*RI DNA fragments in pEC2.6 and pEC3.6 were subcloned for sequencing. DNA sequencing was performed with suitable synthetic primers by the dideoxy termination method as described above.

Immunization of mice and *E. chaffeensis* challenge. The rP28 band in SDS-PAGE was excised, minced, and mixed with an equal volume of Freund's incomplete or complete adjuvant. Nine male BALB/c mice (6 weeks old) were divided into two groups. Five mice were intraperitoneally immunized a total of four times at 10-day intervals: twice with a mixture of the minced gel with rP28 (30 to 40 μg of protein per mouse each time) and incomplete adjuvant and twice with a mixture of the recombinant protein (the same amount as before) and complete adjuvant. Four mice were intraperitoneally injected with a mixture of the minced gel without protein and the respective adjuvants. For ehrlichia challenge, approximately 10^7 DH82 cells heavily infected with *E. chaffeensis* were disrupted by sonication in serum-free Dulbecco modified Eagle medium (GIBCO-BRL) and centrifuged at 200 × *g* for 5 min. The supernatant was diluted to a final volume of 5 ml, and 0.3 ml was inoculated intraperitoneally into each mouse 10 days after the last immunization.

Detection of *E. chaffeensis* 16S rDNA in *Ehrlichia*-challenged mice. At day 5 postchallenge, approximately 1 ml of blood from each mouse was collected in an EDTA tube. Total DNA was prepared from 0.2 ml of the buffy coat from the blood with a QIAamp blood kit (Qiagen, Inc., Chatsworth, Calif.) and was used as the template for PCR detection of *E. chaffeensis* 16S ribosomal DNA (rDNA). PCR detection with primers HE1 (5′-CAATTGCTTATAACCTTTTGGTTATAAAT-3′) and HE3 (5′-TATAGGTACCGTCATTATCTTCCCTAT-3′), which yield a 389-bp fragment specific to *E. chaffeensis* 16S rDNA (4), was performed as described previously (39). The procedure allows detection from ≥10 pg of genomic DNA from purified *E. chaffeensis*.

Sequence analysis. Nucleotide sequences were analyzed with the DNASIS program (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). A homology search was carried out with the GenBank, Swiss Plot, PDB, and PIR databases by using the software basic local alignment search tool (2) in the BLAST network service (National Center for Biotechnology Information, Bethesda, Md.). Phylogenetic analysis was performed by using the PHYLIP software package (version 3.5) (17). An evolutionary distance matrix, generated by using the Kimura formula (17) in the PROTDIST, was used for construction of a phylogenetic tree by using unweighted pair-group method analysis (17). The data were also examined by using parsimony analysis (PROTPARS in PHYLIP). A bootstrap analysis was carried out to investigate the stability of randomly generated trees by using SEQBOOT and CONSENSE in the same package.

Nucleotide sequence accession numbers. The nucleotide sequences of the *p28* gene and its gene copies have been assigned GenBank accession numbers U72291 and AF021338, respectively.

RESULTS

Identification of major outer membrane proteins of *E. chaffeensis*. The ehrlichial outer membrane fraction was prepared from Percoll-purified *E. chaffeensis* by Sarkosyl treatment. Transmission electron microscopy revealed that the purified ehrlichial fraction consists of a mixture of small electron-dense and large light forms with slight disintegration of the inner membrane (Fig. 1A). The host inclusion membrane was not found with the purified ehrlichiae. Various sizes of membrane vesicles (<1 μm) without significant ribosomes or nuclear materials were observed in the Sarkosyl-insoluble fraction prepared from the purified organism (Fig. 1B). Succinic dehydrogenase (an inner membrane marker enzyme of gram-negative bacteria) activity was less than the detection limit (1 nmol/min/mg of protein) in the Sarkosyl-insoluble fraction, compared to approximately 10 nmol/min/mg of protein in the Percoll-purified organisms, suggesting that the insoluble fraction consisted primarily of the outer membrane of *E. chaffeensis*.

Analysis of the Sarkosyl-soluble and insoluble fractions of *E. chaffeensis* by SDS-PAGE suggested that proteins in the 30-kDa range in the insoluble fraction represent the major outer membrane proteins of this organism (Fig. 2A). *E. canis* was antigenically cross-reactive with *E. chaffeensis* (7, 30). A similar result was obtained with *E. canis* by the same procedure (Fig. 2B). These findings indicate that the 30-kDa-range proteins represent the major outer membrane proteins of these two *Ehrlichia* spp. Since it was impossible to resolve overlapping protein bands in the 30-kDa range by conventional SDS-

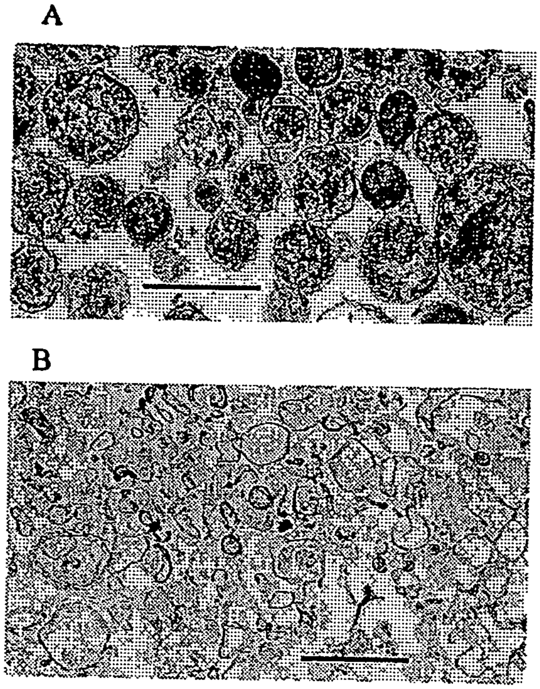

FIG. 1. Transmission electron microscopy of Percoll-purified *E. chaffeensis* (A) and of the insoluble precipitate after 0.1% Sarkosyl treatment of the organism (B). Note outer membrane vesicles of various sizes in panel B. Bars, 1 μm.

PAGE, RdSDS-PAGE was performed, and at least five proteins (P23, P25, P27, P28, and P29, designated based on the molecular sizes in Fig. 2C) of the outer membrane fraction of *E. chaffeensis* were resolved. The N-terminal amino acid sequences of all these proteins were chemically determined, and that of P28 was found to be homologous to that of *C. ruminantium* MAP-1 (36) by a BLAST search.

Cloning, sequencing, and expression of a gene (*p28*) encoding *E. chaffeensis* P28. A 0.8-kb *p28* gene, amplified by PCR, was cloned and sequenced as described in Materials and Methods. The 0.8-kb DNA fragment, cloned in pCRII*p28*, had an open reading frame (ORF) of 756 bp encoding a 251-amino-acid protein (including both PCR primer regions) with a molecular mass of 27,685 Da. *E. coli* transformed with pET29*p28* expressed a 31-kDa rP28 (Fig. 3A), which was larger than the native P28 because of the fusion protein. rP28 has an additional 35-amino-acid sequence including the S.Tag peptide (20) derived from a pET expression vector at the N terminus. The serum from a patient with clinical signs of human ehrlichiosis reacted strongly to rP28 (31 kDa) in *E. coli*, to P28 and P29 in *E. chaffeensis*, and also to P30 in *E. canis* (Fig. 3B). The rabbit anti-rP28 antibody recognized not only rP28 (31 kDa) and P28 but also P29 and P25 of *E. chaffeensis* and P30 of *E. canis* (Fig. 3C), indicating that P28 shares antigenic epitopes with these proteins.

Immunoelectron microscopy. Transmission immunoelectron microscopy with colloidal gold-conjugated protein G and rabbit anti-rP28 antibody revealed gold particles bound to the *E. chaffeensis* surface (Fig. 4). The distribution of the particles was random and close to the surface, and they appeared as if almost embedded in the membrane, suggesting that the antigenic epitope only slightly protrudes on the surface. Nonetheless, the antigenic epitope was surface exposed and thus could be recognized by rabbit anti-rP28 antibody. No gold particles were observed on the host cytoplasmic membrane or *E. chaffeensis* incubated with normal rabbit serum.

Identification and characterization of genomic copies of the *E. chaffeensis p28* gene. Genomic Southern blot analysis with several restriction enzymes resulted in one or more DNA fragments of *E. chaffeensis* which could hybridize to the ^{32}P-labeled

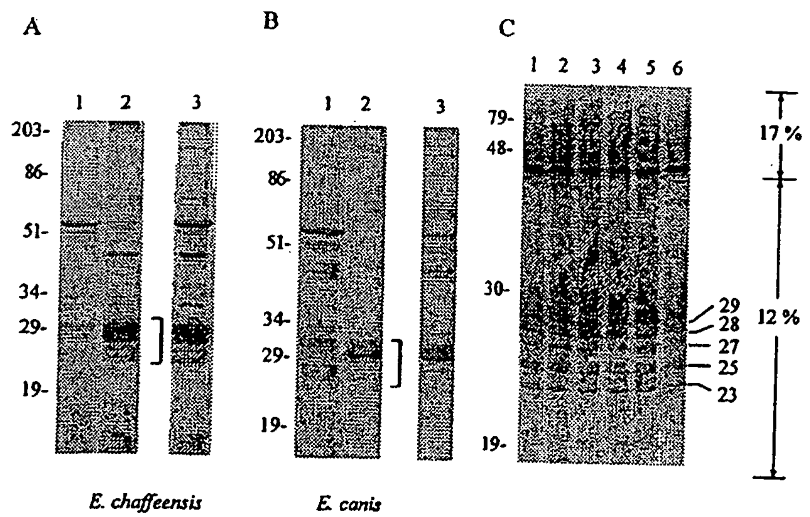

FIG. 2. SDS-PAGE patterns of the insoluble precipitate and the soluble supernatant fraction after 0.1% Sarkosyl treatment of purified *E. chaffeensis* (A) and *E. canis* (B) and RdSDS-PAGE of major proteins in the 30-kDa range resolved from the Sarkosyl-insoluble pellet of *E. chaffeensis* (C). (A) Lanes: 1, Sarkosyl-soluble supernatant; 2, Sarkosyl-insoluble precipitate enriched with outer membrane; 3, Percoll gradient-purified *E. chaffeensis*. (B) Lanes: 1, Sarkosyl-soluble supernatant; 2, Sarkosyl-insoluble precipitate; 3, purified *E. canis*. Both gels were stained with Coomassie blue. Brackets indicate a 30-kDa cluster of major outer membrane proteins. (C) The separation gel consisted of a 17% gel on top of a 12% gel. The Sarkosyl-insoluble precipitate prepared from purified *E. chaffeensis* was blotted onto a ProBlot membrane and stained with amido black. The protein bands present in six lanes of the membrane were excised, and the N-terminal amino acid sequence of each protein was analyzed. Numbers on the right or left of panels indicate molecular masses in kilodaltons.

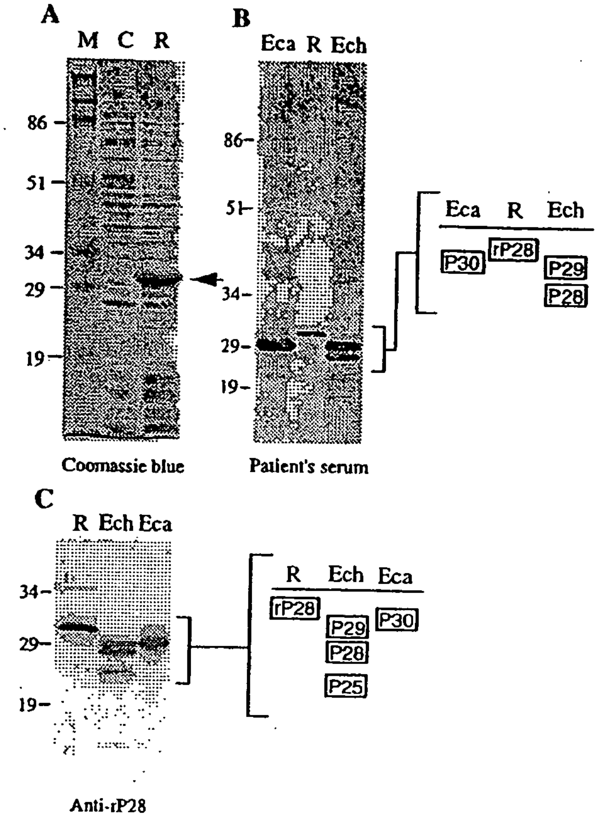

FIG. 3. Overexpression of the *E. chaffeensis p28* gene (A) and Western blot analysis with convalescent-phase serum from a human ehrlichiosis patient (B) and with a rabbit anti-rP28 antibody (C). Lanes: M, molecular size markers; C, pET29a-transformed *E. coli* (negative control); R, pET29*p28*-transformed *E. coli* (recombinant) (arrowhead, rP28); Eca, purified *E. canis*; Ech, purified *E. chaffeensis*. Dominant protein antigens with the molecular masses of P25 to P30, and rP28 (31 kDa), are schematically shown. Numbers indicate molecular masses in kilodaltons.

p28 gene probe (Fig. 5). The restriction enzymes used do not cut within the *p28* gene portion of the pCRII*p28* insert, and therefore, this result indicates that multiple genes homologous to the *p28* gene are present in the ehrlichial genome. *Xba*I, *Bgl*II, and *Kpn*I produced two bands, *Spe*I generated three bands, and *Eco*RV and *Pst*I produced multiple bands with different densities. *Eco*RI generated a broad band of 2.5 to 4 kb. These *p28*-homologous genes are designated the *omp-1* (for outer membrane protein 1) family.

Four DNA fragments from 2.6 to 3.6 kb were cloned from the *Eco*RI- and *Pst*I-digested genomic DNA of *E. chaffeensis* by colony hybridization with the radiolabeled *p28* gene probe. The DNA inserts of the two recombinant clones pEC3.6 and pPS2.6 overlapped as shown in Fig. 6. Sequencing revealed one 5′-truncated ORF of 243 bp (designated *omp-1A*) and five complete ORFs of 836 to 861 bp (designated *omp-1B* to *omp-1F*) that were tandemly arranged and homologous to the *p28* gene, but not identical, in the ehrlichial genomic DNA of 6,292 bp. The intergenic spaces were 581 bp between *omp-1A* and *omp-1B* and 260 to 308 bp among the others. Putative promoter regions and ribosome-binding sites were identified in the noncoding regions upstream from the start codon of each gene.

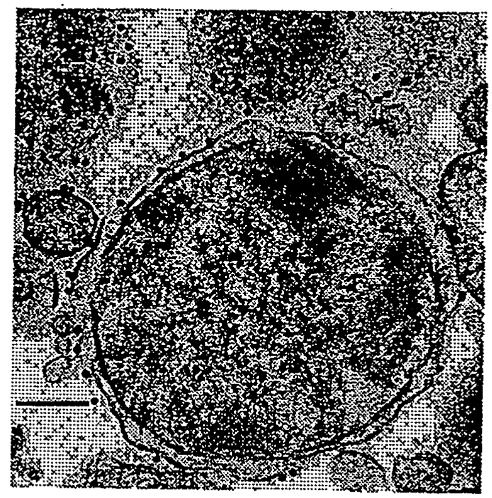

FIG. 4. Transmission electron microscopy of *E. chaffeensis* immunogold labeled with a rabbit anti-rP28 antibody. Protein G-gold particles (20 nm) are localized on the surface of the organism. Bar, 0.1 μm.

Structures of proteins encoded by the genes of the *E. chaffeensis omp-1* family. Five complete *omp-1* gene copies (*omp-1B* to *omp-1F*) encode 279- to 287-amino-acid proteins with molecular masses of 30,320 to 31,508 Da. *omp-1A* encodes an 82-amino-acid partial protein (9,243 Da) which lacks the N-terminal region. The 25-amino-acid sequence at the N termini of OMP-1B to OMP-1F (encoded by *omp-1B* to *omp-1F*, respectively) is predicted to be a signal peptide, because three carboxyl-terminal amino acids of the signal peptides (Ser-X-Ala in OMP-1B, Leu-X-Ser in OMP-C, and Ser-X-Ser in OMP-1D and OMP-1F) are among the preferred amino acid sequences of the signal peptidase at its processing site (26). The molecular masses of the mature OMP-1B to OMP-1F calculated based on the predicted amino acid sequences are 28,181 Da for OMP-1B, 27,581 Da for OMP-1C, 28,747 Da for OMP-1D, 27,776 Da for OMP-1E, and 27,933 Da for OMP-1F. The estimated isoelectric points of these proteins are 4.76 to 5.76.

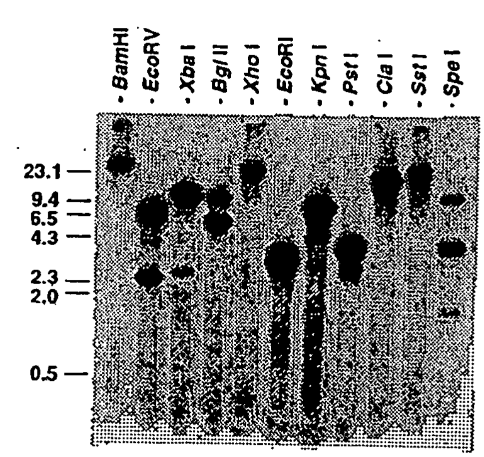

FIG. 5. Genomic Southern blot analysis of *E. chaffeensis* with a ^{32}P-labeled 0.8-kb *p28* gene probe of the pCRII*p28* insert. Numbers indicate molecular sizes in kilobases.

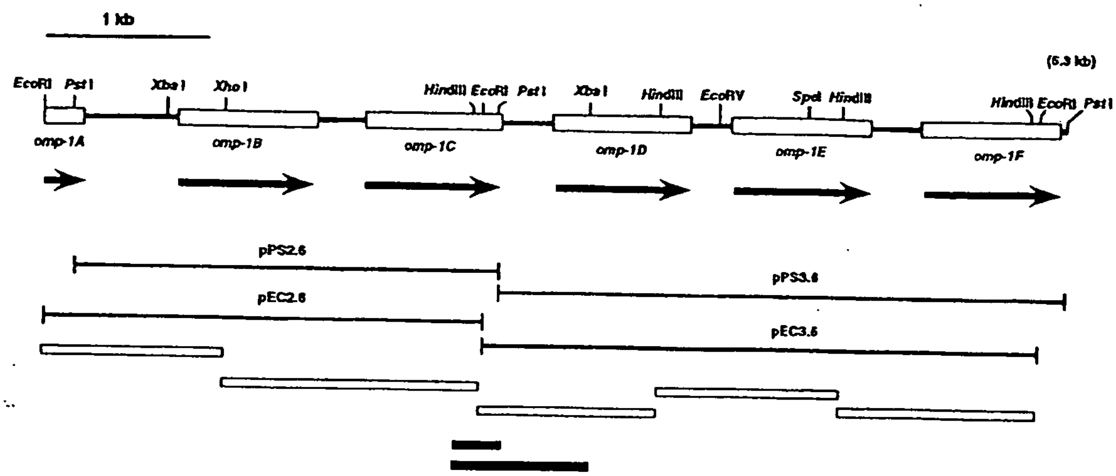

FIG. 6. Restriction map of 6.3 kb of *E. chaffeensis* genomic DNA including the *omp-1* gene copies. The four DNA fragments pPS2.6, pPS3.6, pEC2.6, and pEC3.6 were cloned from the genomic DNA. Recombinant plasmid pPS2.6 has a sequence overlapping that of pEC3.6. The black boxes at the bottom show PCR-amplified fragments from the genomic DNA for confirmation of the overlapping area. Open boxes at the top indicate ORFs of *omp-1* gene copies, with directions indicated by arrows. Open boxes at the bottom show DNA fragments subcloned for DNA sequencing.

Alignment of predicted amino acid sequences of the *E. chaffeensis* OMP-1 proteins, along with that of *C. ruminantium* MAP-1 (36), which is related to the OMP-1 family, revealed substitutions or deletions of one or several contiguous amino acid residues throughout the molecules. The significant differences in sequences among the aligned proteins are seen in the regions designated semivariable (SV) and hypervariable (HV) in Fig. 7. Computer analysis for hydropathy revealed that protein molecules predicted for all *omp-1* gene copies contain alternative hydrophilic and hydrophobic motifs which are characteristic of transmembrane proteins. HV1 and HV2 were found to be located in the hydrophilic regions (data not shown).

An amino acid sequence in HV1 (underlined within OMP-1F in Fig. 7) was identical to the chemically determined N-terminal amino acid sequence (NSPENTFNVPNYSFK) of the *E. chaffeensis* native P23 protein, suggesting that P23 is derived from the *omp-1F* gene. Amino acid sequences identical

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                                 SV                                                            HV1
OMP-1F   MNCKKFFITT TLVSLMSFLP GISFSDAVQN DNVG-GN---.-FYISGKYVP SVSHFGVFSA KQ-----ERN TTTGVFGLKQ DWDGSTISKN SPENTFNVPN  90
OMP-1E   .......... A......... ......P..G ..IS-..----.-..V....M. .A....M... .E-----.K. P.VALY.... ..E.-IS.SS HND.H..NKG  89
OMP-1D   ...E...... A.TL...... ...L..P..D ..IS-..--- -.......M. .A........ .E-----... ..V....IE. ...RCV..RT TLSDI.T...  90
OMP-1C   .......... A.ALP..... ..LL.EP..D .S.S-..--- -.......M. .A........ .E-----.K. P.VALY.... ..N.-VSASS HADAD..NKG  89
OMP-1B   ..Y..I.VSS A.I....I.. YQ..A.P.TS NDT.INDSRE G....V..N. .I...RK... EEAPINGNTS I.KK.....K .------GDI AQSAN..RTD  94
P28      ---------- ---------- -----.PA-G SGIN-..--- -.......M. .A........ .E-----... ..V....... N....A..NS ..NDV.T.S.  64
MAP-1    .....I...S T.I..V.... .V....VI.E E.NPV.S--- -V...A..M. TA....KM.I .E-----DSR D.KA.....K ....VKTPSG NTNSI.TEKD  91
OMP-1A   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                      HV2
OMP-1F   YSFKYENNPF LGFAGAVGYL MNGPRIELEM SYETFDVKNQ GNNYKNDAH- -KYYALTH-- NSGGKLSNAG DKFVFLKNEG LLDISLMLNA CYDVISEGIP 186
OMP-1E   .......... ......I..S .G...V.F.V .......... .........- -R.C..GQ-- -QDNSGIPKT S.Y.L..S.. .....F.... ...I.N.S.. 184
OMP-1D   ........L. S.....I..S .D.......V ...A...... ......E..- -R....S.LL GTETQIDG.. SAS...I... ...K.F.... .......... 188
OMP-1C   .......... ......I..S .G.....F.V .......... .G.......- -R.C..DR-- -KASSTNATA SHY.L..... .......... ...V...... 184
OMP-1B   PALEFQ..LI S..S.SI..A .D.......A AYQK..A..P D..DT.SGDY Y..FG.SR-- ----EDAI.D K.Y.V..... ITFM...V.T ...ITA..V. 188
P28      .......... ......I..S .D.......V .......... ......E..- -R.C..SH-- ..AADM.S.S NN........ .....F.... ....VG.... 160
MAP-1    .......... .........S ........P.V .......R.P .G.......- -M.C.----L DTASSSTAGA TTS.MV...N .T........ ...DMLD.M. 185
OMP-1A   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                                HV3
OMP-1F   FSPYICAGVG TDLISMFEAI NPKISYQGKL GLSYSISPEA SVFVGGHFHK VIGNEFRDIP AMIPSTSTLT GN-HF----T IVTLSVCHFG VELGGRFNF  280
OMP-1E   L......... .........T .......... ......N... ...I...... .......... TLKAFVTSS- -ATPDL---A .......... I........  278
OMP-1D   ........I. I..V...... .......... ....P..... ...I...... .......... T....E.A.A .KGNYP---A ....D.FY.. I........QL 286
OMP-1C   .......... .......... .......... ......N... .......... .A.......S TLKAPATPSS AATPDL---A T......... .........  280
OMP-1B   .I..A..... A...NV.KDF .L.F.....I .I..P.T..V .A.I..YY.G ....N.NK.. VIT.VVLEGA PQTTS----A L..IDTGY.. G.V.V..T.  283
P28      ........I. ...V.M...T .......... .......... ...I...... .......... TI..TG...A .KGNYP---A ..I.D..... I......V.  256
MAP-1    V...V...I. ...V.VDN.T ...L...... .I....N... .I.I.....R ......K..A TSKVF..SGN ASSAVSPGFA SAI.D..... I.I....V.  284
OMP-1A   ---------- ----EF.D.L HV.FA..... .I..QLFTKV NL.LD.YY.Q ....Q.KNLN VNHVY.LKES PKVTS----A VA..DIAY.. G.V.I..T.  81
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FIG. 7. Amino acid sequence alignment of seven *E. chaffeensis* OMP-1 proteins and *C. ruminantium* MAP-1. Aligned positions of amino acids identical to those in OMP-1F are shown with dots. The sequence of *C. ruminantium* MAP-1 is from the report of Van Vliet et al. (36). Gaps (indicated by dashes) were introduced for optimal alignment of all proteins. Bars indicate a semivariable region (SV) and three hypervariable regions (HV1, HV2, and HV3). The chemically determined N-terminal amino acid sequence of *E. chaffeensis* P23, which was identical to the amino acid sequence of OMP-1F, is underlined. The arrowhead shows the putative cleavage site of the signal peptide.

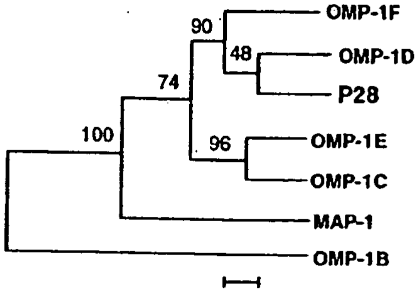

FIG. 8. Phylogenetic relationship among six members of the *E. chaffeensis* OMP-1 family and *C. ruminantium* MAP-1. The evolutionary distance values were determined by the method of Kimura (17), and the tree was constructed by unweighted pair-group method analysis. The scale bar shows 5% divergence in the amino acid sequences. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown.

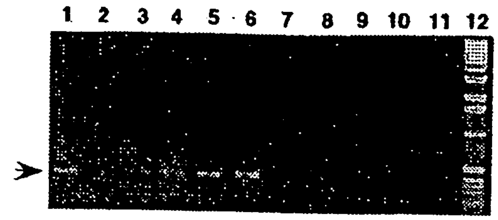

FIG. 9. PCR detection of *E. chaffeensis* 16S rDNA fragment in the blood of *E. chaffeensis*-challenged mice previously immunized with rP28 or nonimmunized. Template DNAs were prepared from blood buffy coats (0.2 ml) of all challenged mice. The arrow shows the *E. chaffeensis*-specific 16S rDNA fragment (389 bp) obtained by PCR amplification. Lanes: 1, positive control (with a total DNA from DH82 cells infected with *E. chaffeensis* as the template); 2, negative control (PCR without template); 3 to 6, nonimmunized mice; 7 to 11, immunized mice; 12, 1-kb DNA ladder marker (GIBCO).

to the N-terminal sequences of P25, P27, and P29 were not found among those from *omp-1* gene copies cloned in this study (data not shown).

Similarities among amino acid sequences of the *E. chaffeensis* OMP-1 proteins. The amino acid sequences of five mature proteins without signal peptides (OMP-1C to OMP-1F and P28) were similar to one another (71 to 83%), but the sequence of OMP-1B was dissimilar to those of the five proteins (45 to 48%). The amino acid sequences of the five proteins showed an intermediate degree of similarity to that of *C. ruminantium* MAP-1 (59 to 63%), but the similarity between those of OMP-1B and *C. ruminantium* MAP-1 was low (45%). In Fig. 8, these relations are shown in a phylogenetic tree based on the amino acid sequence alignment. Three proteins (P28, OMP-1D, and OMP-1F) and two proteins (OMP-1C and OMP-1E) formed two separate clusters. OMP-1B was located distantly from these two clusters. *C. ruminantium* MAP-1 was positioned between OMP-1B and other members of the OMP-1 family.

Protection against *E. chaffeensis* challenge in rP28-immunized mice. To investigate whether immunization with rP28 induces protection against *E. chaffeensis* infection, five mice were immunized with rP28 and four mice were inoculated with acrylamide gel without the recombinant protein (control). Before challenge, all five immunized mice had a titer of 1:160 against *E. chaffeensis* antigen by indirect immunofluorescence assay and all four nonimmunized mice were negative. Protection was assessed by PCR detection of *E. chaffeensis* 16S rDNA in the buffy coat of blood collected from the mice at 5 days postchallenge. *E. chaffeensis* can transiently establish infection in BALB/c mice. The infection is spontaneously cleared, as *E. chaffeensis* cannot be reisolated in cell culture at day 10 postinfection (28). Day 5 is the optimum time at which establishment of ehrlichial infection can be examined by PCR without the influence of residual DNA from the ehrlichiae used as the challenge before the spontaneous clearance of organisms takes place. The *E. chaffeensis*-specific DNA fragment was observed in all nonimmunized mice but not in any immunized mice, indicating that immunization with rP28 apparently protects mice from ehrlichial infection (Fig. 9) and suggesting that the P28 is a potential protective antigen.

DISCUSSION

The outer membrane is the site where the host-ehrlichia interaction takes place. So far, the outer membrane fraction has not been prepared from any *Ehrlichia* spp.; consequently, the protein composition of the outer membrane is unknown. Using a Sarkosyl method, we identified five major proteins (P23 to P29) in the insoluble fraction of *E. chaffeensis*. Three of the five (P25, P28, and P29) were found to be antigenically cross-reactive by using anti-rP28 antibody, and the antigenic epitopes were surface located in *E. chaffeensis* as demonstrated by transmission immunoelectron microscopy. These observations, in addition to results of analysis by transmission electron microscopy and examination of succinic dehydrogenase activity in the Sarkosyl-insoluble fraction, support the usefulness of the Sarkosyl procedure for preparation of a fraction enriched in the outer membrane of *E. chaffeensis*. Like for *O. tsutsugamushi* (25), the concentration of Sarkosyl required for *E. chaffeensis* was lower than those required for other facultative intracellular bacteria (6, 18, 37).

This is the first report in which the major outer membrane proteins of *E. chaffeensis* in the 30-kDa range are identified and characterized at the molecular genetic and protein sequence levels. We and other investigators previously reported protein antigens of *E. chaffeensis* ranging from 22 to 30 kDa (7–10, 13, 30, 40). The two dominant antigens, P28 and P29 in the current study, seem to correspond, respectively, to two proteins of 28 and 30 kDa reported by Rikihisa et al. (30) and to two proteins of 28 and 29 kDa reported by Chen et al. (7). In both previous studies, the antigens were recognized predominantly by convalescent-phase sera from human ehrlichiosis patients. P28 and P29 may also correspond, respectively, to proteins of 29 and 30 kDa reported by Chen et al. (8), both of which were recognized by the 7C1-B and 3C7 MAbs. The current study, using the anti-rP28 antibody, and the study of Chen et al. (8), using the MAbs, indicated that P28 (the current study) and the 29-kDa protein (8) share antigenic epitopes with P29 (the current study) and the 30-kDa protein (8), respectively. In the current study, P25, P28, and P29 were recognized by the anti-rP28 antibody. It is unknown whether *E. chaffeensis* P23, P25, and P27 (the current study) are identical to the three antigens of 22, 26, and 28 kDa recognized by MAb 1A9 (8). The *E. canis* 30-kDa protein was recognized by the antibody to rP28 of *E. chaffeensis* (the current study) and by the 7C1-B MAb to *E. chaffeensis* (8, 10). The 32-kDa MAP-1 of *C. ruminantium* (36) showed amino acid sequence similarity to all members of the *E. chaffeensis* OMP-1 family. *C. ruminantium* MAP-1 also was cross-reactive to a 27-kDa protein of *E. canis* (22), although it is unknown whether the 27-kDa protein is identical to P30 of *E. canis* in the current study. By 16S rDNA sequence comparison, *E. chaffeensis*, *E. canis*, and *C. ruminantium* are closely related (12). Consequently, the 30-kDa-range proteins in the

OMP-1 family may be common antigens among the three species in the tribe *Ehrlichieae*.

By using the PCR-amplified *p28* gene as a probe, six similar genes were identified in the *E. chaffeensis* genome. Genomic Southern blotting results suggest the presence of additional *omp-1* gene copies. However, the precise number of copies cannot be determined, since restriction site polymorphism in the gene copies may result in the production of several bands from a single copy.

We think that P23 is generated from the OMP-1F by a specific processing, rather than by nonspecific degradation during the preparation of the outer membrane fraction, since there was no difference in protein profiles determined by SDS-PAGE among several batches of purified organisms or outer membrane fractions prepared in the presence or absence of proteinase inhibitors.

Recently, in *A. marginale*, which is related to *E. chaffeensis* as determined by 16S rDNA sequencing (12), two multigene families were found (1, 27). A family of *msp-2* genes that encode a 36-kDa major surface protein constitute a minimum of 1% of the genome and are distributed widely throughout the chromosome. In addition, strain variations of the *msp-2* copies were demonstrated (27). A family of *msp-3* gene copies that encode a 63-kDa major surface protein are also distributed widely throughout the chromosome. *msp-3-12* has a DNA sequence area homologous to that of *msp-2* within the ORF of *msp-3-12*. *msp-3-11* and *msp-3-19* have a DNA sequence area homologous to that of *msp-2* outside ORFs (1). The *omp-1* gene family of *E. chaffeensis* is different from these gene families of *A. marginale*. First, the ORFs of *omp-1* gene copies were tandemly arranged in the genome. Second, amino acid sequences among the *omp-1* copies have a greater variation than the reported variations of *msp-2* copies of *Anaplasma*. The similarities were 45 to 83% among six *omp-1* copies, whereas the similarity is 95% between two *msp-2* copies (15). Strain variability may also exist in *E. chaffeensis*, since the reactivities of protein antigens to MAb 7C1-B are different among three strains (8, 10).

In phylogenetic analysis, three proteins (P28, OMP-1D, and OMP-1F) belong to the same cluster. P23 (most likely derived from the *omp-1F* gene), which was identified in the *E. chaffeensis* outer membrane fraction, also belongs to this cluster. It is unknown whether *omp-1D* and other gene copies in different clusters are silent genes. These genes at least are not actively expressed in the population of *E. chaffeensis* from which our specimen was prepared, since the products from the *omp-1* gene family, except for P23, P25, P28, and P29, were not recognized in the Sarkosyl-insoluble outer membrane fraction.

We demonstrated that rP28 protected mice from *E. chaffeensis* infection or accelerated the spontaneous clearance of *E. chaffeensis*, suggesting that this or other *omp-1*-related proteins may be a protective antigen. Further molecular genetic studies are required to elucidate the mechanisms of the antigenic polymorphism or possible antigenic variation, i.e., whether selective expression of the *omp-1* gene copies is regulated at the transcriptional level or by recombination events (gene conversions) among the unique gene repertoire, such as in the cases of the pili of *Neisseria gonorrhoeae* (19), *vmp* of *Borrelia hermsii* (5), and *vls* of *Borrelia burgdorferi* (43).

ACKNOWLEDGMENTS

This work was supported by grant RO1 AI33123 from the National Institutes of Health. Norio Ohashi was a recipient of a 1995 postdoctoral fellowship from The Ohio State University, and Ning Zhi was a recipient of a 1995 graduate student fellowship from The Ohio State University.

We thank John Lowbridge for his assistance in N-terminal amino acid sequencing, Quin Lu for her assistance in DNA sequencing, Andrea Nicastro for her help in ehrlichial cultivation, and Jason Mott and Roy Barnewall for their editorial assistance.

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Editor: J. G. Cannon

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1998, p. 2671–2680
0095-1137/98/$04.00+0

Vol. 36, No. 9

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Cloning and Characterization of Multigenes Encoding the Immunodominant 30-Kilodalton Major Outer Membrane Proteins of *Ehrlichia canis* and Application of the Recombinant Protein for Serodiagnosis

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Received 2 March 1998/Returned for modification 7 April 1998/Accepted 16 June 1998

A 30-kDa major outer membrane protein of *Ehrlichia canis*, the agent of canine ehrlichiosis, is the major antigen recognized by both naturally and experimentally infected dog sera. The protein cross-reacts with a serum against a recombinant 28-kDa protein (rP28), one of the outer membrane proteins of a gene (*omp-1*) family of *Ehrlichia chaffeensis*. Two DNA fragments of *E. canis* were amplified by PCR with two primer pairs based on the sequences of *E. chaffeensis omp-1* genes, cloned, and sequenced. Each fragment contained a partial 30-kDa protein gene of *E. canis*. Genomic Southern blot analysis with the partial gene probes revealed the presence of multiple copies of these genes in the *E. canis* genome. Three copies of the entire gene (*p30*, *p30-1*, and *p30a*) were cloned and sequenced from the *E. canis* genomic DNA. The open reading frames of the two copies (*p30* and *p30-1*) were tandemly arranged with an intergenic space. The three copies were similar but not identical and contained a semivariable region and three hypervariable regions in the protein molecules. The following genes homologous to three *E. canis* 30-kDa protein genes and the *E. chaffeensis omp-1* family were identified in the closely related rickettsiae: *wsp* from *Wolbachia* sp., *p44* from the agent of human granulocytic ehrlichiosis, *msp-2* and *msp-4* from *Anaplasma marginale*, and *map-1* from *Cowdria ruminantium*. Phylogenetic analysis among the three *E. canis* 30-kDa proteins and the major surface proteins of the rickettsiae revealed that these proteins are divided into four clusters and the two *E. canis* 30-kDa proteins are closely related but that the third 30-kDa protein is not. The *p30* gene was expressed as a fusion protein, and the antibody to the recombinant protein (rP30) was raised in a mouse. The antibody reacted with rP30 and a 30-kDa protein of purified *E. canis*. Twenty-nine indirect fluorescent antibody (IFA)-positive dog plasma specimens strongly recognized the rP30 of *E. canis*. To evaluate whether the rP30 is a suitable antigen for serodiagnosis of canine ehrlichiosis, the immunoreactions between rP30 and the whole purified *E. canis* antigen were compared in the dot immunoblot assay. Dot reactions of both antigens with IFA-positive dog plasma specimens were clearly distinguishable by the naked eye from those with IFA-negative plasma specimens. By densitometry with a total of 42 IFA-positive and -negative plasma specimens, both antigens produced results similar in sensitivity and specificity. These findings suggest that the rP30 antigen provides a simple, consistent, and rapid serodiagnosis for canine ehrlichiosis. Cloning of multigenes encoding the 30-kDa major outer membrane proteins of *E. canis* will greatly facilitate understanding pathogenesis and immunologic study of canine ehrlichosis and provide a useful tool for phylogenetic analysis.

Canine ehrlichiosis is caused by *Ehrlichia canis*, an obligatory intracellular bacterium. It was described originally in Algeria in 1935 (7), and it has now been reported throughout the world and at higher frequency in tropical and subtropical regions (13, 15, 32). Canine ehrlichiosis is characterized by fever, depression, anorexia, and weight loss in the acute phase, with laboratory findings of thrombocytopenia and hypergammaglobulinemia (3, 9). A subclinical phase follows the acute phase (5, 12, 28). In the chronic phase, in addition to the clinical signs and laboratory findings of the acute phase, hemorrhages, epistaxis, edema, and hypotensive shock may occur, which are often exacerbated by superinfection with other organisms (3, 9, 16).

Among several protein antigens of *E. canis*, the proteins in the 30-kDa range were shown to be dominant antigens and consistently recognized by sera from both experimentally and naturally infected dogs in Western blot analysis (14, 25, 26). The proteins of *E. canis* immunologically cross-react with *Ehrlichia chaffeensis* major antigens in the 30-kDa range (25). These *E. canis* and *E. chaffeensis* proteins were found to be major outer membrane proteins (OMPs) (22). Analysis of a 28-kDa major OMP (P28) gene of *E. chaffeensis*, one of the 30-kDa-range antigens, and its gene copies revealed that these proteins are encoded by a polymorphic multigene family (22). The rabbit serum against a recombinant *E. chaffeensis* P28 protein cross-reacted with the 30-kDa protein of *E. canis* (22).

Dot immunoblot assaying has been developed for serodiagnosis of several infectious agents (4, 10, 11, 30). The advantages of the assay are that an expensive instrument is not required and the interpretation of the results is easy, since positive and negative reactions can be distinguished by the naked eye. However, to be used as the antigen, purification of the organism from infected cells is essential, since *E. canis* is an obligate intracellular bacterium. Purification of *E. canis* is time-consuming and expensive, and serial passages of *E. canis*

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in the cell culture may produce batch-to-batch variations. Although, no genes of *E. canis* other than the 16S rRNA gene have thus far been identified, preparation of a recombinant major antigen is expected to greatly improve the serodiagnosis of *E. canis* infection.

In this study, three genes encoding the 30-kDa OMPs from the *E. canis* genome were identified. All were found to be homologous and phylogenetically characterized. A recombinant protein of *E. canis* which was expressed as a fusion protein was found to be highly antigenic. The dot immunoblot assay was developed with the recombinant *E. canis* protein.

MATERIALS AND METHODS

Organisms and purification. *E. canis* Oklahoma and *E. chaffeensis* Arkansas were cultivated in the DH82 dog macrophage cell line and purified by Percoll density gradient centrifugation (22) or Sephacryl S-1000 column chromatography (26).

PCR, cloning, and expression. The sequences of two forward primers, FECH1 and FECH2, were 5'-CGGGATCCGAATTCGG(A/T/G/C)AT(A/T/C)AA(T/C)GG(A/T/G/C)AA(T/C)TT(T/C)TA-3' and 5'-CGGGATCCGAATTCTA(T/C)AT(A/T)AG(T/C)GG(A/T/G/C)AA(A/G)TA(T/C)ATG-3', corresponding to amino acid positions 6 to 12 and positions 12 to 18, respectively, of the mature 28-kDa protein (P28) of *E. chaffeensis* (22). These primers have a 14-bp sequence (underlined) at the 5' end to create an *Eco*RI site and a *Bam*HI site for insertion into an expression vector. The sequence of a reverse primer, REC1, was 5'-ACCTAACTTTCCTTGGTAAG-3', complementary to the DNA sequence corresponding to amino acid positions 185 to 191 of the mature P28 of *E. chaffeensis* (22).

Genomic DNA of *E. canis* was isolated from Percoll gradient-purified organisms as described elsewhere (22). PCR amplification was performed by using a Perkin-Elmer Cetus DNA Thermal Cycler (model 480). The 0.6-kb products were amplified with both primer pairs, FECH1-REC1 and FECH2-REC1, and were cloned in the pCRII vector of a TA cloning kit (Invitrogen Co., San Diego, Calif.). The clones obtained by FECH1-REC1 and FECH2-REC1 were designated pCRII*p30* and pCRII*p30a*, respectively. Both strands of the insert DNA were sequenced by a dideoxy termination method with an Applied Biosystems 373 DNA sequencer.

For expression, the 0.6-kb fragment was excised from the clone pCRII*p30* by *Eco*RI digestion, ligated into *Eco*RI site of a pET29a expression vector, and amplified in *Escherichia coli* BL21(DE3)pLys (Novagen, Inc., Madison, Wis.). The clone (designated pET29*p30*) produced a fusion protein with 35-amino-acid and 21-amino-acid sequences carried from the vector at the N and C termini, respectively.

For purification of a recombinant P30 fusion protein (rP30), the cultivated clone was harvested at 4 h after induction with β-D-thiogalactopyranoside. The recombinant protein in the clone pET29*p30* was enriched in the pellet by three cycles of centrifugation of the lysate after disruption of the transformant by freezing-thawing and sonication. The final pellet was used as a partially purified rP30 antigen. Affinity-purified rP30 protein was obtained by chromatography with His-Bind Resin (Novagen, Inc.). Briefly, after preparation of the partially purified rP30 antigen, the insoluble protein was extracted with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), including 6 M urea. After being applied to a Ni^{+}-conjugated column, the recombinant protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 6 M urea. The refolding of the purified protein was achieved by sequential dialysis in 20 mM Tris-HCl (pH 7.9) containing 4 and 2 M urea and finally in 20 mM Tris-HCl buffer only and stored at −80°C until use.

Southern blot analysis. Genomic DNA extracted from the Percoll-purified *E. canis* (200 ng each) was digested with restriction enzymes, electrophoresed, and transferred to a Hybond-N^{+} nylon membrane (Amersham, Arlington Heights, Ill.) by a standard method (27). The 0.6-kb DNA inserts containing partial *p30* and *p30a* genes, cloned in pCRII*p30* and pCRII*p30a*, respectively, were separately labeled with [α-^{32}P]dATP by the random primer method with a kit (Amersham), and each labeled fragment was used for Southern blot analysis as a DNA probe. Hybridization was performed at 60°C in Rapid Hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in 0.1× SSC (1× SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate) with 1% sodium dodecyl sulfate (SDS) at 55°C, and the hybridized probes were exposed to Hyperfilm (Amersham) at −80°C.

Cloning and sequencing of 30-kDa protein gene copies from the *E. canis* genomic DNA. The *Hin*dIII DNA fragment, which was detected by genomic Southern blot analysis as described above, was inserted into pBluescript II KS(+) vectors, and the recombinant plasmids were introduced into *E. coli* DH5α. By using the colony hybridization method (27), two positive clones which contained ehrlichial DNA fragments of 3.6 and 7.3 kb were isolated with the ^{32}P-labeled inserts of pCRII*p30* and pCRII*p30a* as probes, respectively. DNA sequencing was performed with suitable synthetic primers by the dideoxy termination method described above.

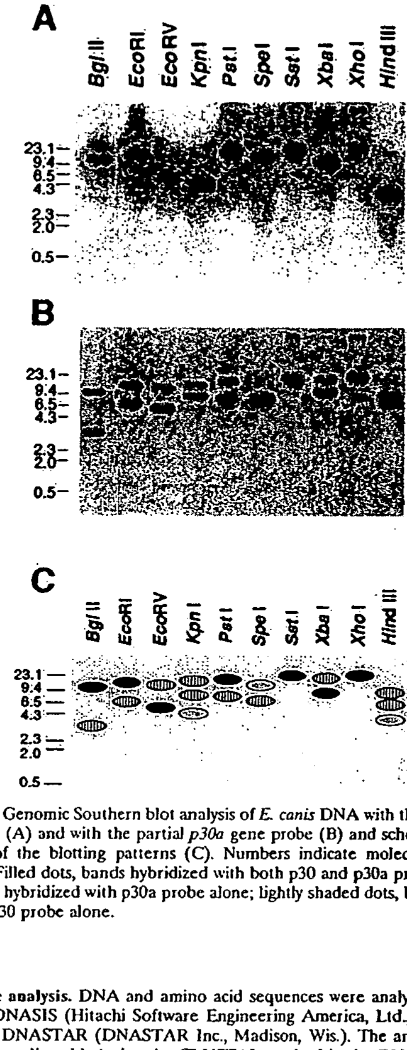

FIG. 1. Genomic Southern blot analysis of *E. canis* DNA with the partial *p30* gene probe (A) and with the partial *p30a* gene probe (B) and schematic representation of the blotting patterns (C). Numbers indicate molecular sizes in kilobases. Filled dots, bands hybridized with both p30 and p30a probes; striped dots, bands hybridized with p30a probe alone; lightly shaded dots, bands hybridized with p30 probe alone.

Sequence analysis. DNA and amino acid sequences were analyzed with the programs DNASIS (Hitachi Software Engineering America, Ltd., San Bruno, Calif.) and DNASTAR (DNASTAR Inc., Madison, Wis.). The amino acid sequences were aligned by using the CLUSTAL method in the DNASTAR program. Phylogenetic analysis was performed by using the PHYLIP software package (version 3.5) (8). An evolutionary distance matrix, generated by using the Kimura formula in the program PROTDIST in the package, was used for construction of a phylogenetic tree by using the unweighted pair-group method of analysis (8). The data were examined by using parsimony analysis (PROTPARS in the PHYLIP). A bootstrap analysis was carried out to investigate the stability of randomly generated trees by using SEQBOOT and CONSENSE in the same package.

Dog plasma and mouse serum. Totals of 34 and 8 dog blood samples with heparin or EDTA were obtained from the Southwest Veterinary Diagnostic Center (Phoenix, Ariz.) and at the Ohio State University Veterinary Teaching Hospital, respectively. All blood specimens collected were centrifuged at 250 × *g* for 5 min, and the plasma samples were used for this study. For Western blot analysis, these plasma samples were preabsorbed three times with pET29a-transformed *E. coli* at 4°C overnight prior to use. For preparation of the mouse anti-rP30 serum, a male mouse (BALB/c) was intraperitoneally immunized a total of four times at 10-day intervals, once with an equal mixture of the affinity-purified rP30 (30 μg of protein) and Freund's complete adjuvant (Sigma) and three times with an equal mixture of the protein (30 μg) and Freund's incomplete adjuvant. The mouse was sacrificed 7 days after final immunization, and the serum was prepared from blood collected from the heart.

IFA and Western blot analysis. Indirect fluorescent antibody assays, (IFA) and Western blot analysis were performed by a procedure described elsewhere (25). Fluorescein isothiocyanate-conjugated goat anti-dog immunoglobulin G (IgG; Organon Teknika Co., Durham, N.C.) and peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were used at dilutions of 1:200 for IFA and 1:2,000 for Western blot analysis, respectively, as secondary antibodies.

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                         ▼ SV                                                                                    HV1
P30       MNCKRFFIAS ALISLMSFLP SVSFSESIHE DNIN-GN--- -FYISAKYMP SASHFGVFSV KE-----EKN TTTGVFGLKQ DWDGATIKDA SSSHTIDPST IFSISNYSFK  100
P30-1     ....KIL.TT ......YSI. .I...DT.QD G.MG-..--- -....G..V. .V....S..A ..-----.SK S.V......H ....SP.LKN KHAD------ -.TVP....R   93
P30a      .KY.KT.TVT ..VL.T..TH FIP.YSPARA ST.H--.--- -....G.... T.....I..A ..-----.QS F.KVLV..D. RLSHNI.NNN DTAKS----- -LKVQ.....   93
P28       ..Y.KV..T. .....I.S.. G....DPA-G SG..-..--- -....G.... .........A ..-----.R. ..V....... N...SA.SNS .PND------ V.TV......   93
OMP-1F    ....K...TT T.V....... GI...DAVQN ..VG-..--- -....G..V. .V.......A .Q-----.R. .......... ....S..SKN .PEN------ T.NVP.....   94
OMP-1E    ....K...TT ..V....... GI...DPVQG ...S-..--- -..V.G.... ......M..A ..-----... P.VALY.... ..E.-ISSSS HNDN------ H.NNKG....   93
OMP-1D    ...EK...TT ..TL...... GI.L.DPVQD ...S-..--- -....G.... .........A ..-----.R. ..V....IE. . .RCV.SRT TL.D------ ..TVP.....   94
OMP-1C    ....K...TT ..ALP..... GILL..PVQD .SVS-..--- -....G.... .........A ..-----... P.VALY.... ..N.-VSASS HADA------ D.NNKG....   93
OMP-1B    ..Y.KI.VS. .......I.. YQ..ADPVTS NDTGINDSRE G....V..N. .I...RK..A E.APINGNTS I.KK.....K .--------- ---GD.AQ.A N.NRTDPALE   98
MAP-1     ....KI..T. T....V.... G....DV.Q. E.NPV.S--- -V........ T.....KM.I ..-----DSR D.KA.....K ....VKTPSG NTNS------ ..TEKD....   95
OMP-1A    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                 HV2
P30       YENNPFLGFA GAIGYSMGGP RVEFEVSYEI FDVKNQGNSY KNDAH--KYC ALSRHTGGMP Q-AGHQ---- --NKFVFLKN EGLLDISLMI NACYDITIDS MPFSPYICAG  201
P30-1     .......... .......... .I...I...A ....SPNIN. Q....--R.. ...H..SAAM E-.------- --D....... ...I....A. ......IN.K V.V.......  191
P30a      .K........ R.....I.NS .I.L...H.. ..T..P..N. L..S.--... ...HGSHICS DGNSGDWYTA KTD...L... .....V.F.L .......TEK ..........  201
P28       .......... .......D.. .I.L.....T ........N. ..E..--R.. ...H--NSAA DMSSAS---- --.N...... .......F.L .....VVGEG I.........  193
OMP-1F    .......... ..V..L.N.. .I.L.M...T ........N. ......--.Y ..TH--NSGG KLSNAG---- --D....... .........L .....VISEG I.........  194
OMP-1E    .......... .......... .........T ........N. ......--R.. ..GQ---QDN SGIPKT---- --S.Y.L..S .......F.L ......INE. I.L.......  192
OMP-1D    ....L.S... .......D.. .I.L.....A ........N. ..E..--R.Y ...HLL.TET .ID.AG---- --SAS...I. .....K.F.L .....VISEG I.........  196
OMP-1C    .......... .......... .I.......T .......GN. ......--R.. ..D.---KAS STNATA---- --SHY.L... .........L .....VVSEG I.........  192
OMP-1B    FQ..LIS..S .S...A.D.. .I.L.AA.QK ..A..PD.ND T.SGDYY..F G...EDAIA- ---------- -DK.Y.V... ..ITFN...V .T.....AEG V..I..A...  196
MAP-1     .......... ..V...N... .I.......T ...R.P.GN. ......--M.. ..----DTAS SSTAGA---- --TTS.MV.. .N.T.....L ......ML.G ..V...V...  193
OMP-1A    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                                 HV3
P30       IGSDLVSMFE TTNPKISYQG KLGVSYSISP EASVFVGGHP HRVIGNEFRD IPA---ITPA GATEIKGTQF -TTVTLNICH FGLELGGRPTF     288
P30-1     ..T..I.... A.S....... ...I....N. .T...I.... ..I.....R. ...----.V.S NS.T.S.P.. -A.....V.. .........N.     278
P30a      ..T..I.... ..QN...... ...LN.T.NS RV...A.... .K.......G ..T---LL.D .SNIKVQQS- -A....DV.. .....I.S..F.   287
P28       ..T....... A......... ...L...... .....I.... .K......R. ..T---.I.T .S.LAGKGNY PAI.I.DV.. ..I......V.     281
OMP-1F    V.T..I.... AI........ ...L...... .......... .K......R. ..----MI.S TS.LTGN-H. -.I...SV.. ..V......N.     280
OMP-1E    V.T..I.... A......... ...L....N. .....I.... .K......R. ..T---LKAF VTSS--A.PD LAI...SV.. ..I......N.     278
OMP-1D    ..I....... AI........ ...L..P... .....I.... .K......R. ..T---MI.S ESALAGKGNY PAI...DVFY ..I......N.QL  286
OMP-1C    V.T..I.... AI........ ...L....N. .......... .K.A....R. .ST---LKAF ATPSSAA.PD LA....SV.. ..V......N.     280
OMP-1B    V.A..INV.K DF.L.F.... .I.I..P.T. .V.A.I..YY .G....N.NK ..VITPVVLE ..PQTTS--- -AL..IDTGY ..G.V.V....    283
MAP-1     ..T....VIN A....L.... ...I....N. ...I.I.... .......... .ATSKVF.SS .NASSAVSPG FASAI.DV.. ..I.I....V.    284
OMP-1A    ------EF.D ALHV.FA... ...I..QLFT KVNL.LD.YY .Q....Q..N LNVNHVY.LK ESPKVTS--- -AVA..D.AY ..G.V.I....     81
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FIG. 2. Amino acid sequence alignment of P30, P30-1, and P30a of *E. canis*, seven members of *E. chaffeensis omp-1* multigene family (P28 and OMP-1A to OMP-1F), and MAP-1 of *C. ruminantium* (Senegal strain). The sequences of the *E. chaffeensis omp-1* gene family and MAP-1 are from the reports of Ohashi et al. (22) and Van Vliet et al. (31), respectively. Aligned positions of identical amino acids with P30 of *E. canis* are indicated by dots. Gaps (indicated by dashes) were introduced for optimal alignment of all proteins. Bars indicate an SV and three HVs (HV1, -2, and -3). The arrowhead indicate the putative cleavage site of the signal peptide.

Dot immunoblot assay. Protein concentrations of purified *E. canis* and recombinant rP30 antigens were determined by a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as a standard. These antigens in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl) were adsorbed onto a nitrocellulose membrane by using a dot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.), blocked for 30 min with TBS containing 2% milk, air dried, and stored at −20°C until use. For immunoassays, the antigen bound to a nitrocellulose strip was incubated with the plasma samples, which were diluted 1:1,000 in TBS containing 2% milk for 1 h at room temperature. After being washed three times with TBS containing 0.05% Tween 20 (T-TBS), the strip was incubated with peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard) at a dilution of 1:2,000 in TBS containing 2% milk. After being washed with T-TBS, the antibody-bound dot was detected by immersing the strip in a developing solution (0.3% 3,3′-diaminobenzidine tetrahydrochrolide [Nacalai Tesque, Inc., Kyoto, Japan] and 0.05% hydrogen peroxide in 70 mM sodium acetate [pH 6.2]). The color intensity was analyzed by using background correction in image analysis software (ImageQuaNT program; Molecular Dynamics, Sunnyvale, Calif.).

GenBank accession number. The DNA sequences of the *p30*, *p30a*, and *p30-1* genes of *E. canis* have been assigned GenBank accession numbers AF078553, AF078555, and AF078554, respectively.

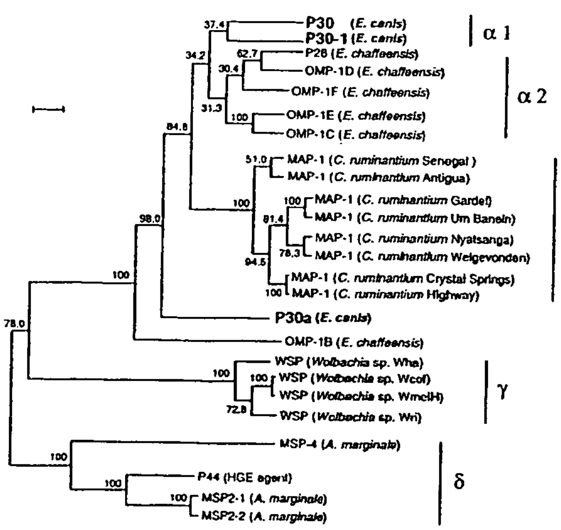

FIG. 3. Phylogenetic classification among P30, P30-1, and P30a of *E. canis* and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Evolutionary distance values were determined by the method described by Kimura, and the tree was constructed by the unweighted pair-group method of analysis. Scale bar indicates 10% divergence in amino acid sequences. Bootstrap values from 100 analyses are shown at the branch points of the tree. Bars with symbols indicate representative clusters. The GenBank accession numbers of the major OMP gene sequences of the organisms used in the analysis are as follows: P28 (*E. chaffeensis*), U72291; OMP-1B to OMP-1F (*E. chaffeensis*), AF021338; MAP-1 (*C. ruminantium* Senegal strain), I40882, MAP-1 (*C. ruminantium* Antigua strain), U50830; MAP-1 (*C. ruminantium* Gardel strain), U50832; MAP-1 (*C. ruminantium* Um Banein strain), U50835; MAP-1 (*C. ruminantium* Nyatsanga strain), U50834; MAP-1 (*C. ruminantium* Welgevonden strain), U49843; MAP-1 (*C. ruminantium* Crystal Springs strain), U50831; MAP-1 (*C. ruminantium* Highway strain), U50833; WSP (*Wolbachia* sp. Wha strain), AF020068; WSP (*Wolbachia* sp. Wcof strain), AF020067; WSP (*Wolbachia* sp. WmelH strain), AF020066; WSP (*Wolbachia* sp. Wri strain), AF020070; MSP-4 (*A. marginale*), Q07408; MSP2-1 (*A. marginale*), U07862; MSP2-2 (*A. marginale*), U36193; and P44 (HGE agent), AF059181.

RESULTS

Cloning and sequencing of three 30-kDa protein gene copies of *E. canis*. Two 0.6-kb DNA fragments containing partial *p30*

TABLE 1. Similarities among amino acid sequences of *E. canis* P30, P30-1, and P30a; *E. chaffeensis omp-1* family (OMP-1B to OMP-1F and P28); *C. ruminantium* MAP-1; *Wolbachia* spp. WSP; HGE agent P44; and *A. marginale* MSP-4, MSP2-1, and MSP2-2

Protein	% Amino acid sequence similarity and evolutionary distance for the following proteins[a]:										
	P30	P30-1	P30a	P28	OMP-1F	OMP-1E	OMP-1D	OMP-1C	OMP-1B	MAP-1 (Senegal)	MAP-1 (Antigua)
P30		80.2	70.8	80.6	80.5	78.6	77.8	77.5	63.2	75.4	76.2
P30-1	0.38628		71.6	79.8	81.7	78.7	78.3	77.3	63.2	74.7	75.6
P30a	0.60811	0.60559		73.9	72.1	73.3	71.2	72.1	58.8	67.2	67.8
P28	0.36288	0.40582	0.50899		85.7	82.3	86.3	81.1	63.6	76.4	77.5
OMP-1F	0.37862	0.36209	0.59907	0.27551		83.4	84.9	83.0	63.2	75.4	75.8
OMP-1E	0.41426	0.42866	0.52142	0.35465	0.32640		81.7	90.1	63.4	76.8	78.1
OMP-1D	0.45193	0.46724	0.61591	0.25793	0.28867	0.36288		81.5	63.2	73.5	74.5
OMP-1C	0.45426	0.48329	0.57469	0.39823	0.34577	0.18285	0.37688		62.4	76.0	77.5
OMP-1B	0.89214	0.87276	0.99793	0.81397	0.83501	0.82982	0.84498	0.89516		62.7	63.2
MAP-1 (Senegal)	0.50490	0.51605	0.76041	0.46987	0.50383	0.46987	0.57453	0.50564	0.92668		93.9
MAP-1 (Antigua)	0.47614	0.50899	0.74635	0.46755	0.52220	0.46096	0.57153	0.48952	0.88842	0.09122	
MAP-1 (Gardel)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.13499	0.11546
MAP-1 (Crystal Springs)	0.55702	0.53478	0.78883	0.52220	0.56563	0.49693	0.59089	0.53368	0.93601	0.13657	0.14142
MAP-1 (Highway)	0.52891	0.52047	0.76041	0.49443	0.54364	0.46987	0.57594	0.50564	0.93601	0.12383	0.12856
MAP-1 (Nyatsanga)	0.50593	0.49693	0.76544	0.49196	0.53368	0.46755	0.57296	0.48952	0.91855	0.13077	0.11963
MAP-1 (Um Banein)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.12658	0.11963
MAP-1 (Welgevonden)	0.52629	0.50383	0.74708	0.49877	0.53368	0.47419	0.60290	0.48952	0.92979	0.16080	0.14519
WSP (Wha)	1.57097	1.66864	1.78274	1.59949	1.50435	1.38174	1.61950	1.45510	1.41776	1.58338	1.48404
WSP (Wcof)	1.46262	1.62571	1.62571	1.55195	1.40877	1.29961	1.60271	1.41762	1.33110	1.55897	1.53089
WSP (WmelH)	1.48165	1.64952	1.64952	1.54244	1.39991	1.31514	1.59304	1.43572	1.34750	1.54961	1.49206
WSP (Wri)	1.46435	1.66864	1.70518	1.55687	1.46526	1.27219	1.57654	1.39076	1.32111	1.53292	1.47465
P44	1.77884	1.84928	2.04164	1.56146	1.74020	1.64702	1.64376	1.64702	1.64566	1.57894	1.63909
MSP-4	1.37226	1.39399	1.62744	1.38660	1.45473	1.36494	1.45413	1.47002	1.34294	1.23482	1.31702
MSP2-1	1.50323	1.53992	1.90757	1.40230	1.59474	1.53455	1.40877	1.50435	1.52758	1.53992	1.54847
MSP2-2	1.52476	1.53992	1.87540	1.40230	1.57132	1.53455	1.40877	1.50435	1.55019	1.51796	1.52616

[a] Values in the upper right half are percent amino acid sequence similarities; those in the lower left half are evolutionary distances.

and *p30a* genes, amplified by PCR, were cloned and sequenced as described in Materials and Methods. The 0.6-kb DNA, cloned in pCRII*p30*, had an open reading frame (ORF) of 579 bp encoding a 193-amino-acid protein with a molecular mass of 21,175 Da. Another 0.6-kb fragment, cloned in pCRII*p30a*, had an ORF of 564 bp encoding a 188-amino-acid protein with a molecular mass of 21,042 Da. The DNA and predicted amino acid sequences of the partial *p30a* gene were similar but not identical to those of the partial *p30* gene. Genomic Southern blot analysis of *E. canis* digested with several restriction enzymes revealed one and two DNA fragments which could strongly hybridize to the partial *p30* and *p30a* gene probes, respectively (Fig. 1). These restriction enzymes used do not cut within the *p30* and *p30a* gene probes, and, therefore, the result with the *p30a* probe indicates that another gene homologous to the *p30a* is present in the *E. canis* genome. In *Bgl*II, *Eco*RI, and *Pst*I digestion, the *p30* probe hybridized with the upper band of the two *p30a*-hybridized bands. In *Eco*RV and *Xba*I digestion, the *p30* probe hybridized with the lower band of the two *p30a*-hybridized bands. In *Kpn*I, *Spe*I, and *Hin*dIII digestion, the *p30* probe hybridized with one or two bands that were different from the *p30a*-hybridized bands.

Two DNA fragments of 3.6 and 7.3 kb were cloned by colony hybridization with the probes described above from the *Hin*dIII-digested genomic DNA of *E. canis*. Sequencing revealed a complete ORF of 864 bp for the *p30* gene in the 3.6-kb fragment and a complete ORF of 861 bp for *p30a* gene in the 7.3-kb DNA fragment. An additional ORF of 921 bp was found in the 3.6-kb DNA. The DNA sequence of the ORF (designated *p30-1*) was also similar but not identical to those of the *p30* and *p30a* genes. There are two potential start codons in the *p30-1* gene sequence. By comparison with the N-terminal amino acid sequences of *p30* and *p30a* genes, we chose a second ATG as a start codon for phylogenetic analysis. The coding region is 834 bp. The *p30-1* and *p30* genes were tandemly arranged with an intergenic space of 355 bp in the 3.6-kb fragment like the *E. chaffeensis omp-1* family (22). In addition to the result of the genomic Southern blot analysis, this finding showed that at least four homologous genes (*p30*, *p30-1*, *p30a*, and a gene homologous to *p30a*) exist in the *E. canis* genome, suggesting that these genes of *E. canis* are also encoded by a polymorphic multigene family as is the case with *E. chaffeensis* (22).

Structure of proteins encoded by *E. canis* multigenes. Three complete gene copies (*p30*, *p30-1*, and *p30a*) encode 278- to 288-amino-acid proteins with molecular masses of 30,485 to 31,529 Da. The 25-amino-acid sequence at the N termini of P30, P30-1, and P30a (encoded by *p30*, *p30-1*, and *p30a*, respectively) is predicted to be a signal peptide, as described previously (22). The molecular masses of the mature proteins calculated based on the predicted amino acid sequences are 28,750 Da for *p30*, 27,727 Da for *p30-1*, and 29,132 Da for *p30a*.

The predicted amino acid sequences of *E. canis* P30, P30-1, and P30a showed high similarity with those of members in the *E. chaffeensis omp-1* gene family (22) and that of major antigen protein 1 (MAP-1) of *Cowdria ruminantium* (31). These organisms are also serologically cross-reactive (6, 17, 18, 19, 20). The alignment of amino acid sequences of these proteins revealed substitutions or deletions of one or several contiguous amino acid residues throughout the molecules (Fig. 2). The significant differences in sequences among the proteins are observed in the regions designated SV (semivariable region) and HV (hypervariable region). Computer analysis for hydropathy revealed that protein molecules predicted for three *E. canis* gene copies contain alternative hydrophilic and hydrophobic motifs which are characteristic of typical transmembrane proteins. HV1 and HV2 were located in the hydrophilic regions (data not shown).

TABLE 1—*Continued*

% Amino acid sequence similarity and evolutionary distance for the following proteins:													
MAP-1 (Gardel)	MAP-1 (Crystal Springs)	MAP-1 (Highway)	MAP-1 (Nyatsanga)	MAP-1 (Um Banein)	MAP-1 (Welgevonden)	WSP (Wha)	WSP (Wcof)	WSP (WmelH)	WSP (Wri)	P44	MSP-4	MSP2-1	MSP2-2
76.4	74.5	75.4	75.8	76.4	75.2	44.4	44.6	44.4	44.4	19.5	45.6	27.8	27.4
74.7	73.9	74.3	74.7	74.7	74.5	44.0	45.1	44.8	44.6	20.5	47.6	29.3	29.1
67.6	65.9	66.5	66.7	67.6	67.2	41.5	43.2	42.9	42.5	19.5	43.1	24.2	24.2
75.8	74.5	75.4	75.2	75.8	74.9	44.0	44.8	44.8	44.6	22.5	46.9	29.7	29.5
74.5	73.3	73.9	73.9	74.5	73.9	44.6	45.9	45.9	45.3	21.1	46.2	27.8	27.8
76.2	75.4	76.2	76.0	76.2	75.8	45.7	46.9	46.7	46.9	22.0	47.5	28.2	28.0
74.1	73.1	73.5	73.3	74.1	72.4	43.6	44.2	44.2	44.2	22.0	46.0	29.9	29.7
75.8	74.5	75.4	75.6	75.8	75.6	45.3	46.1	45.9	46.1	22.0	46.6	28.6	28.4
63.6	63.2	63.2	63.2	63.6	62.9	45.5	45.1	44.8	45.5	19.1	45.8	26.9	26.5
91.4	90.7	91.4	91.6	91.8	90.1	44.6	45.1	45.1	45.1	21.8	48.8	28.0	28.0
91.8	90.7	91.4	91.6	91.6	90.3	44.8	45.1	45.3	45.3	21.8	48.0	28.0	28.0
	92.2	92.8	94.9	99.6	93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.12928		98.9	93.1	92.4	93.1	43.4	43.4	43.4	43.2	20.0	46.1	26.7	26.7
0.11692	0.01764		93.7	93.1	93.7	43.8	43.8	43.8	43.6	20.2	46.5	27.2	27.2
0.08788	0.11285	0.10076		94.5	95.4	43.8	43.8	43.8	43.8	20.5	46.7	28.0	27.8
0.00693	0.12514	0.11285	0.09570		93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.11966	0.11285	0.10076	0.08014	0.11966		44.2	44.0	44.0	44.0	20.2	46.5	27.8	27.6
1.51972	1.73099	1.65953	1.64538	1.51972	1.58048		86.1	86.1	90.3	12.5	42.5	22.9	22.7
1.47157	1.59304	1.53089	1.55897	1.47157	1.52893	0.27243		98.3	90.9	13.6	42.1	24.0	24.0
1.46262	1.58338	1.52153	1.54961	1.46262	1.51972	0.26757	0.03029		90.7	13.6	42.3	23.8	23.8
1.44526	1.64362	1.57654	1.53292	1.44526	1.50279	0.18429	0.17605	0.17691		13.6	43.2	24.0	23.8
1.62813	1.74020	1.71093	1.68253	1.62813	1.71093	2.06354	2.15803	2.14440	2.09032		25.7	45.5	45.2
1.33120	1.35101	1.30992	1.31112	1.33120	1.33120	1.72157	1.96007	1.90199	1.72157	1.20170		35.6	34.9
1.50996	1.57836	1.53304	1.46817	1.50996	1.48884	1.70865	1.79325	1.81891	1.72741	0.83164	1.20880		95.6
1.50996	1.55543	1.51116	1.46817	1.50996	1.48884	1.70865	1.75923	1.78382	1.72741	0.84284	1.23930	0.05064	

Phylogenetic relationship among the three *E. canis* 30-kDa proteins and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Recently, several major OMP genes which are closely related to the *E. canis* 30-kA protein have been cloned from rickettsiae (2, 21–24, 31, 34). The phylogenetic tree consisting of 25 major OMPs of the organisms including P30, P30-1, and P30a of *E. canis* was constructed from the estimated evolutionary distances (Fig. 3). The overall pattern of the tree reflects the result based on 16S rRNA gene sequence analysis of the rickettsiae. The 23 representatives, except for *E. canis* P30a and *E. chaffeensis* OMP-1B, are divided into four groups as follows: *E. canis* and *E. chaffeensis*, group α; *C. ruminantium*, group β; *Wolbachia* sp., group γ; and the agent of human granulocytic ehrlichiosis (HGE) and *Anaplasma marginale*, group δ. Group α formed a subcluster of *E. canis* P30 and P30-1 (group α1), which was separated from another subcluster composed of five *E. chaffeensis* OMPs (group α2). The similarities between P30 and P30-1 of *E. canis* in group α1, between groups α1 and α2, between groups α1 and β, between groups α1 and γ, and between groups α1 and δ were 80.2%, 77.3 to 80.6%, 73.9 to 76.4%, 44.0 to 45.1%, and 19.5 to 47.6%, respectively (Table 1). On the other hand, *E. canis* P30a and *E. chaffeensis* OMP-1B were far from group α and were located between groups β and γ. The similarities between *E. canis* P30a and group α1, between P30a and group α2, between P30a and group β, between P30a and group γ, and between P30a and group δ were 70.8 to 71.6%, 71.2 to 73.9%, 65.9 to 67.8%, 41.5 to 43.2%, and 19.5 to 43.1%, respectively.

Expression of the *E. canis p30* gene. The clone pET29*p30* produced a 249-amino-acid fusion protein with a molecular mass of 27,316 Da (Fig. 4A). The recombinant protein (rP30) with minimum *E. coli* contamination detectable was obtained in the pellet by centrifugation of the lysate of the transformant (Fig. 4B [partially purified antigen]). The rP30 protein further purified by affinity chromatography from this preparation had a single band on SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4B [affinity-purified antigen]). The immunoreactions of *E. canis* rP30 with a total of 42 clinical dog plasma specimens were examined. The IgG-IFA titers of 29 plasma samples were 1:20 to 1:10,480. The remaining plasma samples were IFA negative (<1:20). Western blot analysis revealed that all IFA-positive plasma samples recognized the partially purified rP30 fusion protein (27 kDa) and a 30-kDa protein of

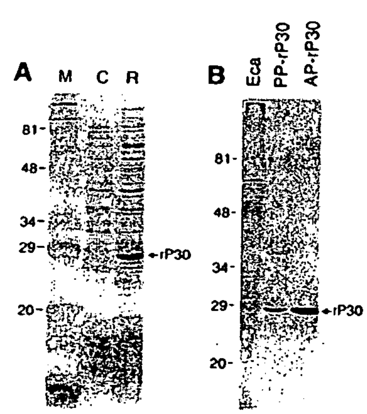

FIG. 4. SDS-PAGE profiles of a recombinant clone expressing P30 of *E. canis* (A) and the purified recombinant protein (B). Gels were stained with Coomassie blue. Lanes: M, molecular size markers; C, pET29-transformed *E. coli* (negative control); R, pET29*p30*-transformed *E. coli* (recombinant); Eca, purified *E. canis*; PP-rP30, partially purified rP30 fusion protein of *E. canis*; and AP-rP30, affinity-purified rP30 fusion protein. The recombinant rP30 protein is indicated by the arrow. The numbers on the left of each panel indicate molecular masses in kilodaltons.

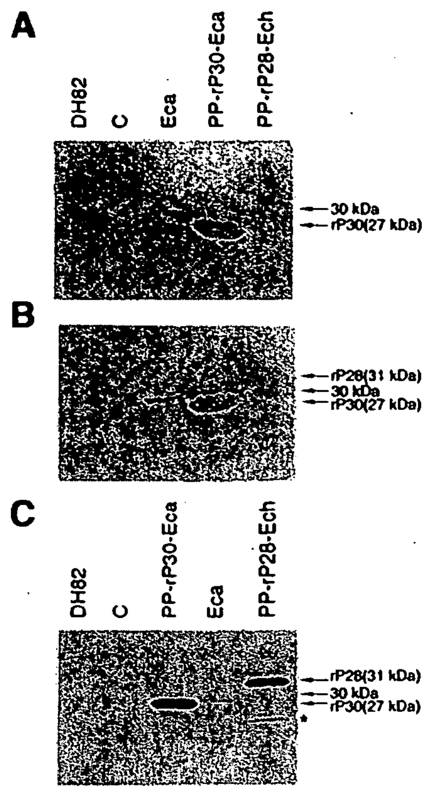

FIG. 5. Western blot analysis with clinical dog plasma with canine ehrlichiosis (A and B) and mouse anti-rP30 serum (C). (A) Dog plasma with a 1:40 IFA titer against *E. canis*; (B) dog plasma with a 1:1,280 IFA titer. Lanes: DH, DH82 dog macrophage cell (negative control); C, a pET29-transformed *E. coli* (negative control); Eca, purified *E. canis* (reactive 30-kDa protein is indicated by arrows in each panel); PP-rP30-Eca, a partially purified rP30 fusion protein (27 kDa) of *E. canis*; and PP-rP28-Ech, a partially purified rP28 fusion protein (31 kDa) of *E. chaffeensis* (22). Another smaller reactive band which may be a degradation product of rP28 of *E. chaffeensis* is indicated by an asterisk.

purified *E. canis* (one of the blots is shown in Fig. 5A), but none of 13 negative plasma samples reacted with any proteins of partially purified rP30 and purified *E. canis* (data not shown). Eight of the 29 positive plasma samples reacted weakly with recombinant P28 fusion protein (rP28 [31 kDa]) of *E. chaffeensis* (22) (one of the blots is shown in Fig. 5B), but the remaining plasma samples did not. A mouse anti-rP30 serum which was prepared by immunization with the affinity-purified antigen reacted with the rP30 antigen, a 30-kDa protein of purified *E. canis*, and an rP28 of *E. chaffeensis* (Fig. 5C). Another smaller band which was observed with *E. chaffeensis* rP28 may be a degradation product of rP28 (asterisk in Fig. 5C), since the plasma sample did not react with *E. coli* proteins. These results showed that rP30 of *E. canis* is highly antigenic and that the antigenic epitope is expressed.

Dot immunoblot assay with the purified whole organism antigen and the recombinant antigen. (i) Optimum amount of antigen per dot. Western blot analysis and dot immunoblot assaying in the preliminary experiments supported the interpretation that there are no significant differences between affinity-purified and the partially purified rP30 in specificity and sensitivity (data not shown). If partially purified recombinant protein is suitable for serodiagnosis, it will be more cost-effective. By dot immunoblot assaying we examined in detail whether partially purified rP30 is suitable as an antigen for serodiagnosis.

Nitrocellulose strips having serially diluted purified *E. canis* or partially purified rP30 antigen of *E. canis* were reacted at a 1:1,000 dilution with dog plasma samples with different IFA titers against *E. canis*, and the color intensities of the reaction of each dot were compared (Fig. 6). Dots of 0.01 to 1 μg of the purified organisms (Fig. 6A) or dots of 0.025 to 1 μg of rP30 (Fig. 6B) that reacted with positive plasma samples (>1:20 in IFA titer) were clearly distinguishable from those that reacted with negative plasma samples (<1:20) by the naked eye. There was no nonspecific reaction with the negative plasma samples when purified *E. canis* was used as an antigen; however, a weak nonspecific reaction with IFA-negative plasma was observed in dots of 0.25 to 1 μg of partially purified rP30 antigen. Based on these results, the optimum amounts of antigens per dot were determined to be 1 and 0.5 μg for antigen proteins of purified *E. canis* and partially purified rP30, respectively. These results show that the partially purified recombinant protein is apparently sufficient as an antigen for serodiagnosis.

(ii) Optimum dilution of antiserum. The immunoreactivities of plasma at dilutions of 1:300, 1:1,000, and 1:3,000 were examined with nitrocellulose strips of the purified *E. canis* an-

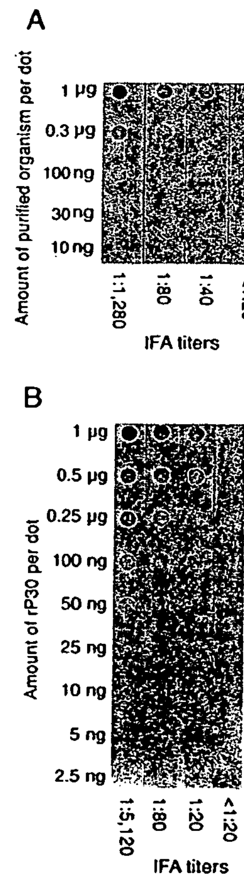

FIG. 6. Optimum amount of antigens for dot blot assaying with purified *E. canis* antigen (A) or partially purified rP30 antigen (B). Purified organism antigen (10 ng to 1 μg) or rP30 antigen (2.5 ng to 1 μg) was blotted onto the nitrocellulose sheet, reacted with each plasma at a 1:1,000 dilution as primary antibody, and reacted with secondary antibody (peroxidase-conjugated affinity-purified anti-dog IgG antibody) at a 1:2,000 dilution.

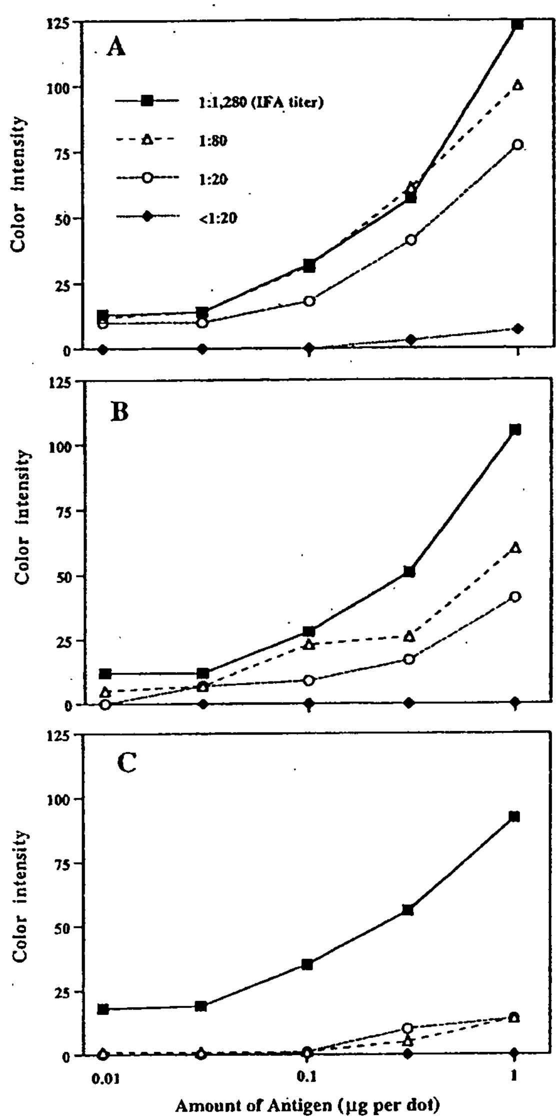

FIG. 7. Optimum plasma dilutions for dot blot assay. Purified *E. canis* antigen was blotted as described in the legend to Fig. 6. The antigens were incubated with plasma at dilutions of 1:300 (A), 1:1,000 (B), and 1:3,000 (C). The plasma samples used were the same as those used for Fig. 6A. The color intensity of each dot was determined by using the image software program (ImageQuaNT).

tigen as shown in Fig. 6A. The color intensity values were plotted in graphs (Fig. 7). At a 1:300 dilution (Fig. 7A), color development occurred in the dots having an antigen greater than 0.3 μg per dot with IFA-negative plasma. At a 1:3,000 dilution (Fig. 7C), color intensities of all plasma samples were low, especially in the case of positive plasma samples with low IFA titers (1:20 and 1:80). At a 1:1,000 dilution (Fig. 7B), positive plasma with even the lowest IFA titer (1:20) was distinguishable from IFA-negative plasma by the naked eye, especially with 1 μg of purified *E. canis* antigen per dot (Fig. 6A). The optimum dilution of plasma for testing was, therefore, 1:1,000.

(iii) Examination of clinical dog plasma with purified *E. canis* and partially purified rP30 antigens. A total of 42 clinical dog plasma samples were examined with 1 μg of purified *E. canis* antigen per dot and 0.5 μg of partially purified rP30 antigen per dot (Fig. 8). The plasma samples with higher IFA titers showed a darker reaction with both native and recombinant antigens. The color intensities between plasma with IFA titers of >1:20 and IFA-negative plasma were clearly distinguishable by the naked eye. The correlation between IFA titers and color intensity values by the dot immunoblot assay was examined (Fig. 9). The maximum color intensity values of 13 IFA-negative plasma samples (<1:20) were zero (background) in the purified *E. canis* antigen and 10 in the rP30 antigen. All 29 IFA-positive plasma samples (>1:20) showed color intensity values of greater than 19 in the purified *E. canis* and 18 in the rP30 antigen. The highest color intensity values were 105 in the purified organism and 114 in the rP30 antigen. In both native and recombinant antigens, color intensity values correlated with IFA titers. The correlation coefficients between IFA titers and color intensities of native and recombinant antigens were 0.71 ($P < 0.001$) and 0.68 ($P < 0.001$), respectively. Therefore, it may be possible to estimate an approximate titer of the test serum or plasma by comparing the color densities with those of serially diluted standard serum or plasma.

DISCUSSION

The availability of recombinant immunodominant major surface proteins of *E. canis* will greatly assist in diagnosis and in understanding of the pathogenesis of this intracellular bacterium, such as invasion of host cells, elicitation of the immune response, and mechanisms of the clinical disease. The 30-kDa protein of *E. canis* was shown to be the immunodominant major OMP, which can be recognized by naturally and experimentally infected dog sera (14, 25, 26). Therefore, the 30-kDa protein is the primary recombinant antigen candidate for use in the serodiagnosis of *E. canis* infection. The present study is the first report of molecular characterization of 30-kDa major OMPs of *E. canis*.

Polymorphic multigene families encoding the major OMPs have been identified in *E. chaffeensis*, the HGE agent, and *A. marginale*, which are closely related to *E. canis* based on 16S rRNA gene sequences. Six copies of the *E. chaffeensis p28* gene (*omp-1* gene family) are tandemly arranged with intergenic spaces (22), while copies of the HGE agent *p44* gene and the *A. marginale msp-2* and *msp-3* genes are distributed widely throughout the genomes (1, 23, 34). In this study, the 30-kDa proteins of *E. canis* were also shown to be encoded by a polymorphic multigene family. The two *E. canis* genes are tandemly arranged with an intergenic space as are members of the *E. chaffeensis omp-1* gene family. Although we demonstrated the presence of four gene copies of 30-kDa *E. canis* proteins in the genome, additional gene copies which are tandemly arranged may exist in three genomic *Hin*dIII DNA fragments which hybridized to *p30* and *p30a* probes. Sequence analysis revealed that the 30-kDa proteins (P30, P30-1, and P30a) of *E. canis* had characteristics of the *E. chaffeensis* OMP-1 family (22) and *C. ruminantium* MAP-1 (31). The *C. ruminantium* MAP-1 has been reported to be cross-reactive to a 27-kDa protein of *E. canis* (19), although it is unknown whether the 27-kDa protein is identical to P30, P30-1, or P30a of *E. canis* in this study. Phylogenetic analysis based on the homologs from the closely related rickettsiae revealed that P30 and P30-1 of *E. canis* are present in the same cluster but that P30a is far from the cluster, suggesting that the multigenes encoding the 30-kDa *E. canis* proteins are widely divergent. Interestingly, in the phylogenetic tree, the 30-kDa *E. canis* proteins, the *E. chaffeensis* OMP-1 family, the HGE agent P44, and *A. mar-*

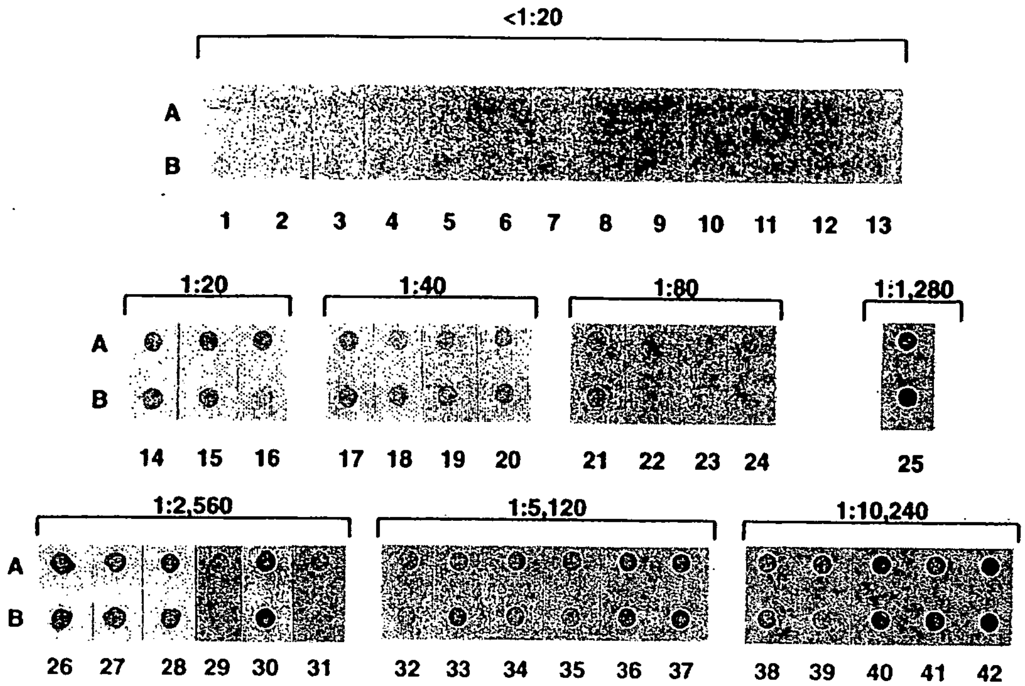

FIG. 8. Reaction profiles of purified *E. canis* antigen (1 μg) (A) and partially purified rP30 antigens (0.5 μg) (B) with 42 plasma samples. Plasma identifications are indicated below each dot. Numbers above brackets indicate the IFA titers of the plasma samples.

ginale MSP-2 are encoded by a polymorphic multigene family as described above. However, *C. ruminantium* MAP-1, *Wolbachia* sp. WSP, and *A. marginale* MSP-4 are encoded by a single gene (2, 21–24, 31). The diversities reported among the *C. ruminantium* MAP-1s and among the *Wolbachia* sp. WSPs are strain variation (2, 24, 31).

Molecular analysis of *E. canis* 30-kDa antigens such as ours is important in understanding the antibody responses of animals, because the antigenic diversity may influence the specificity and sensitivity of the serologic assay. Previously, we observed in the Western blot analysis that acute-phase serum (before 30 days postinoculation) from an *E. canis*-infected dog reacted strongly with a 30-kDa protein but weakly with a 31-kDa protein. However, the reactivity of the chronic-phase serum (after 60 days postinoculation) from the same dog was reversed (strong reaction with the 31-kDa protein and weak reaction with the 30-kDa protein) (14). This might be due to differential expression of the multigene encoding the 30-kDa protein of *E. canis* during infection. Although it is unknown whether the genes of P30, P30-1, and P30a were expressed by *E. canis* in tissue culture or in the infected dog, the recombinant P30 protein constructed in this study expressed the antigenic epitope which can react with all IFA-positive dog plasma samples used, suggesting that the antigenic epitope conserved among the 30-kDa protein gene family is expressed. This strongly supports the idea that rP30 is useful as an antigen for serodiagnosis of canine ehrlichiosis.

For serodiagnosis of canine ehrlichiosis, IFA is widely used. However, a fluorescence microscope and trained personnel are required for this test. Furthermore, cell culture of *E. canis* may produce batch-to-batch variation. A consistent and simple assay that can detect specific antibodies without expensive equipment would be an invaluable aid in serodiagnosis. In the dot immunoblot assay, antibody-positive serum can be distinguished from antibody-negative serum by the naked eye, and if proper color standards are provided, anyone can easily make the final evaluation. The greatest obstacle for the development of this assay is the production of diagnostic antigens sufficient in purity and amount. If recombinant antigens are available, the antigen preparation would be simpler, more consistent, and economical than purified organism antigen preparation. Previously, a dot blot enzyme-linked immunoassay for detecting antibodies to *E. canis* has been reported (4). However, the crude antigens, freed from host cells by freezing-thawing, were used in that study. Neither recombinant antigens nor the purified antigens (such as organisms purified by Sephacryl S-1000 column chromatography) were used. Additionally, that report contains only one page of description without any data. Therefore, we think our dot immunoblot assay using the recombinant 30-kDa antigen of *E. canis* would greatly enhance serodiagnosis of canine ehrlichiosis.

Recognition of the lowest positive IFA titer (1:20) plasma by a dot immunoblot assay with 1 μg or less of protein of the whole organism or the recombinant antigen per dot shows that this assay is as sensitive as IFA. Although the specificity of the test, except for cross-reactivity with *E. chaffeensis*, was not analyzed in this study, as with any other serologic test, dot immunoblot assaying probably cannot distinguish among antigenically cross-reactive members of the tribe *Ehrlichieae*. However, the use of recombinant *E. canis* antigen gave greater sensitivity than the use of recombinant *E. chaffeensis* antigen for serodiagnosis of canine ehrlichiosis. Western blot analysis revealed that 8 of 22 IFA-positive plasma samples slightly cross-reacted with recombinant 28-kDa protein of *E. chaffeensis*. This weak cross-reactivity is not a potential problem for clinics, since treatment is the same for all of the ehrlichial agents.

In dot immunoblot assays of 29 IFA-positive plasma sam-

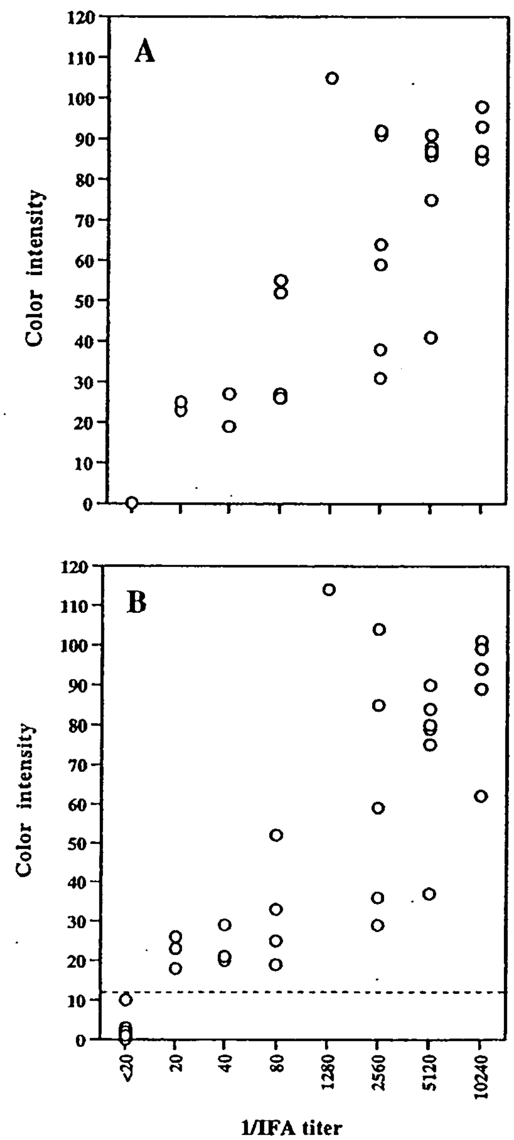

FIG. 9. Correlation between IFA titer (reciprocal dilutions) and color intensity of the dot immunoassay with purified *E. canis* antigen (A) and partially purified rP30 antigen (B). The color intensities of all dots in Fig. 8 were determined and plotted. Each circle represents one plasma specimen (n = 42). The correlation coefficients were 0.71 ($P < 0.001$) for graph A and 0.68 ($P < 0.001$) for graph B. The dashed line in graph B represents the cutoff value, which was determined from the highest color intensity in the immunoreaction with 13 negative plasma samples.

ples, 5 had color intensities of the purified organism antigen greater or lesser than those of the recombinant antigens. Additional major immunodominant proteins of *Ehrlichia* spp. are heat shock proteins (HSPs) (29, 33). Consequently, when anti-HSP antibody or antibody against protein antigen other than P30 is present in the plasma, whole organism antigens would give an immunoreaction stronger than that of the recombinant protein. On the contrary, when anti-P30 antibody is dominant in the plasma, the reaction with the recombinant protein would be stronger than that with the whole organism antigen. More importantly, the recombinant antigen-dot blot assay could clearly detect all of the 29 IFA-positive plasma samples. Furthermore, between native and recombinant antigens, no significant difference was observed in the correlation coefficient between IFA titers and the blot color intensity. Therefore, the rP30 antigen-immunodot blot assay offers advantages over the other serodiagnostic tests in general availability, ease of handling, and accuracy in the serodiagnosis of *E. canis* infection. Additionally, although it was not described in this paper, this *E. canis* recombinant antigen can be applied to enzyme-linked immunosorbent plate assays or other serodiagnostic assays as well.

ACKNOWLEDGMENT

This work was supported by an Ohio State University canine research grant and grant RO1 AI33123 from National Institutes of Health.

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1: AF078553. Reports Ehrlichia canis m...[gi:13512584] Links

Comment Features Sequence

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  JOURNAL   J. Clin. Microbiol. 36 (9), 2671-2680 (1998)
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            Ohio State University, 1925 Coffey Road, Columbus, OH 43210, USA
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1: AF078554. Reports ...[gi:3790556] The record has been replaced by AF078553

Comment Features Sequence

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LOCUS       ECMOMP2                  924 bp    DNA     linear   BCT 26-OCT-1998
DEFINITION  Ehrlichia canis 30-kDa major outer membrane protein (p30-1) gene,
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ACCESSION   AF078554
VERSION     AF078554.1  GI:3790556
KEYWORDS    .
SEGMENT     2 of 3
SOURCE      Ehrlichia canis
  ORGANISM  Ehrlichia canis
            Bacteria; Proteobacteria; alpha subdivision; Rickettsiales;
            Rickettsiaceae; Ehrlichieae; Ehrlichia; canis group.
REFERENCE   1  (bases 1 to 924)
  AUTHORS   Ohashi,N., Unver,A., Zhi,N. and Rikihisa,Y.
  TITLE     Cloning and characterization of multigenes encoding the
            immunodominant 30-kilodalton major outer membrane proteins of
            Ehrlichia canis and application of the recombinant protein for
            serodiagnosis
  JOURNAL   J. Clin. Microbiol. 36 (9), 2671-2680 (1998)
  MEDLINE   98371112
REFERENCE   2  (bases 1 to 924)
  AUTHORS   Ohashi,N., Unver,A., Zhi,N. and Rikihisa,Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JUL-1998) Department of Veterinary Biosciences, The
            Ohio State University, 1925 Coffey Road, Columbus, OH 43210, USA
COMMENT     [WARNING] On Apr 2, 2001 this sequence was replaced by gi:13512584.
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1: AF078555. Reports ...[gi:3790555] The record has been replaced by AF078553

Comment Features Sequence

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DEFINITION  Ehrlichia canis 30-kDa major outer membrane protein (p30a) gene,
            complete cds.
ACCESSION   AF078555
VERSION     AF078555.1  GI:3790555
KEYWORDS    .
SEGMENT     3 of 3
SOURCE      Ehrlichia canis
  ORGANISM  Ehrlichia canis
            Bacteria; Proteobacteria; alpha subdivision; Rickettsiales;
            Rickettsiaceae; Ehrlichieae; Ehrlichia; canis group.
REFERENCE   1  (bases 1 to 864)
  AUTHORS   Ohashi,N., Unver,A., Zhi,N. and Rikihisa,Y.
  TITLE     Cloning and characterization of multigenes encoding the
            immunodominant 30-kilodalton major outer membrane proteins of
            Ehrlichia canis and application of the recombinant protein for
            serodiagnosis
  JOURNAL   J. Clin. Microbiol. 36 (9), 2671-2680 (1998)
  MEDLINE   98371112
REFERENCE   2  (bases 1 to 864)
  AUTHORS   Ohashi,N., Unver,A., Zhi,N. and Rikihisa,Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JUL-1998) Department of Veterinary Biosciences, The
            Ohio State University, 1925 Coffey Road, Columbus, OH 43210, USA
COMMENT     [WARNING] On Apr 2, 2001 this sequence was replaced by gi:13512584.
FEATURES             Location/Qualifiers
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     CDS             1..864
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